γ-Secretase activity is the final cleavage event that releases the amyloid β peptide (Aβ) from the β-secretase cleaved carboxyl-terminal fragment of the amyloid β protein precursor (APP). No protease responsible for this highly unusual, purportedly intramembranous, cleavage has been definitively identified. We examined the substrate specificity of γ-secretase by mutating various residues within or adjacent to the transmembrane domain of the APP and then analyzing Aβ production from cells transfected with these mutant APPs by enzyme-linked immunosorbent assay and mass spectrometry. Aβ production was also analyzed from a subset of transmembrane domain APP mutants that showed dramatic shifts in γ-secretase cleavage in the presence or absence of pepstatin, an inhibitor of γ-secretase activity.

These studies demonstrate that γ-secretase’s cleavage specificity is primarily determined by location of the γ-secretase cleavage site of APP with respect to the membrane, and that γ-secretase activity is due to the action of multiple proteases exhibiting both a pepstatin-sensitive activity and a pepstatin-insensitive activity. Given that γ-secretase is a major therapeutic target in Alzheimer’s disease these studies provide important information with respect to the mechanism of Aβ production that will direct efforts to isolate the γ-secretases and potentially to develop effective therapeutic inhibitors of pathologically relevant γ-secretase activities.

The 4-kDa amyloid β protein (Aβ) that is invariably deposited as amyloid in Alzheimer’s disease (AD) is a normally secreted proteolytic product of the amyloid β protein precursor (APP) (1–3). Generation of Aβ from APP requires two proteolytic events, one at the amino terminus referred to as β-secretase and one at the carboxyl terminus known as γ-secretase (Fig. 1). To date, neither of the proteases responsible for these activities has been definitively identified. Comparison of the soluble Aβ secreted by cells, soluble Aβ in cerebrospinal fluid, and insoluble Aβ isolated from the AD brain has revealed that there are numerous Aβ species with extensive amino- and carboxyl-terminal heterogeneity. The major Aβ species in both conditioned cell culture media and human cerebrospinal fluid is Aβ1–40 (~50–70%) although some Aβ1–42 (5–20%) is also present along with minor amounts of other peptides (e.g. Aβ1–28, Aβ1–33, Aβ1–34, Aβ3–34, Aβ1–37, Aβ1–38, and Aβ1–39) (4–6). The importance of the longer forms of Aβ, in particular Aβ42, has been heightened by the fact that all of the familial Alzheimer’s disease (FAD) linked mutations that have been analyzed result in an increase in the concentration of Aβ42 in a wide variety of model systems (reviewed in Refs. 7 and 8).

Biophysical studies have shown that the longer forms of Aβ aggregate at a much faster rate and at lower concentrations than forms ending at Aβ40 suggesting that alterations in Aβ42 concentration, as occurs in FAD linked forms of the disease, may account for the observation that the longer forms of Aβ are often the initial species deposited in the parenchyma of the AD and Down’s syndrome brain (9–11).

In addition to the β- and γ-secretase activities that generate Aβ, a third proteolytic activity referred to as α-secretase cleaves within the Aβ sequence at Lys16 to release a large secreted derivative (12–14), thus precluding formation of full-length Aβ. Following cleavage of APP at the extracellular/lumenal domain of the APP by either α- or β-secretases, γ-secretase cleavage of the resulting COOH-terminal fragment results in the release of the p3 or Aβ, respectively.

The findings that all of the FAD-linked mutations in APP and the presenilin (PS) genes alter the concentration of Aβ ending at position 42 and, with the exception of the APPK670N,M671L (NL) mutation, appear to act by altering γ-secretase activity makes understanding this activity pivotal to our knowledge of the disease process. While previous studies have shown that β-secretase cleavage, like most proteolytic cleavages, exhibits fairly rigid primary amino acid sequence requirements (15), similar studies of the more complex γ-secretase activity demonstrates fairly loose specificity (16, 17). These studies, however, which either examine effects on total Aβ production (16) or effects of mutations at a single residue, Aβ43 (17), provide no compelling mechanisms for the observed alterations in cleavage associated with FAD-linked mutations.

One of the more unusual aspects of γ-secretase cleavage is that based on hydropathy plots the γ-cleavage sites lie within the putative transmembrane domain (TMD) of the APP (18). Several other proteins, SREBP (19), Notch (20), and mitochondrial inner membrane proteins (21), have been postulated to undergo such intramembranous cleavage. While recent data
for SREBP site two protease cleavage (22) and Notch (20), indicate that the cleavage of these proteins occurs at hydrophobic residues near the transmembrane junction, in no instance has the site of cleavage and the location of the residues with respect to the membrane at the time of cleavage been elucidated. Thus, the concept of intramembranous proteolysis remains controversial. To date, there is no definitive evidence showing that a protease can cleave bonds buried within a membrane.

As a first step toward defining the mechanism of γ-secretase cleavage, we undertook a mutagenesis study in an attempt to define the specificity and structural requirements that produce Aβ species of varying lengths by using a large number of APP TMD mutants. The TMD mutant APPs are shown in Fig. 2. These mutations can be divided into several different categories. 1) Point mutations at positions Aβ41 (I637X based on the APP695 sequence) and Aβ43 (T639X) which correspond to the P1' positions for a γ-secretase cleavage producing Aβ41–40 and Aβ1–42, respectively. 2) Deletion and insertion mutations designed to alter the localization of the γ-secretase cleavage site within the membrane (del and ins). 3) Point mutations that alter the putative membrane stop anchor signal or increase the number of charged residues on the luminal side of the membrane and 4) replacement of residues carboxyl to the normal amino acidic composition 

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant APPs—** A two-step PCR based mutagenesis strategy was employed to generate the various TMD domain mutants. In the first step, the EcoRI/NotI fragment of pcDNA3APP695NL was replaced with a PCR product generated from wild-type APP695 using the forward primer APP-1803 and the reverse primers ∆36 (5’-CATGCGGCCGCTGTCCTTTGAAACCCACATCCTTCGCA-3’), ∆39 (5’-CATGCGGCCGCTGTCCTTTGAAACCCACATCCTTCACCCGCAATCCAGGTCT-3’), and ∆52 (5’-CATGCGGCCGCTGTCCTTTGAAACCCACATCCTTCACCCGCAATCCAGGTCT-3’), respectively, to generate the base constructs pcDNA3APP695NLΔ36, pcDNA3APP695NLΔ39, and pcDNA3APP695NLΔ52. These mutant APPs incorporated a class IIa restriction site, BsmI, 5’ to Aβ26, Aβ39, and Aβ52. When cut with BsmI and NotI the BsmI site is lost, leaving a 5’-4-bp overhang at the 3’ end, effectively truncating the APP. To produce the various TMD mutants in this study, oligonucleotides incorporating the various mutations were generated containing a BsmI site at their 5’ end. PCR products were amplified using various mutant oligonucleotides and a common reverse primer using wild type APP as template. Subsequent cleavage of these products with BsmI and NotI followed by cloning into the appropriate base vectors (pcDNA3APP695NLΔ26, pcDNA3APP695NLΔ39, and pcDNA3APP695NLΔ52) generated the desired mutants with no additional base changes. All PCR reactions were carried out using the High Fidelity PCR Kit from Boehringer Mannheim. All mutant cDNAs were sequenced to ensure that no additional mutations were incorporated. Sequences for all of the PCR primers used are available upon request.

**Transient Transfection of 293 T Cells and Media Collection—** Cells were plated onto 6-well culture dishes (Corning) and grown to 70–80% confluence in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (Life Technologies, Inc.). On the day of transfection, culture media was changed to Opti-MEM (Life Technologies, Inc.). After a short period of equilibration, the media was again changed to fresh Opti-MEM (0.5 ml) and 1.5 μg of DNA and 4.0 μg of DOPSER liposomal transfection reagent (Boehringer Mannheim) were added to each well (premixed) in a volume of 0.5 ml of additional media. After overnight incubation, the media were discarded and replaced with Dulbecco’s modified Eagle’s modified Eagle’s medium (HyClone) supplemented with 10% fetal calf serum (1.0 ml/well). Twenty-four hours later, the media were collected and replaced with serum-free Dulbecco’s modified Eagle’s medium; complete protease inhibitor mixture (Boehringer Mannheim) was added to each sample. The serum-free media were collected 24 h later, and phenylmethylsulfonyl fluoride (to 1 μM) and EDTA (to 5 μM) added to each sample. In both cases, samples were immediately centrifuged at high speed to pellet cellular debris, transferred to clean Microfuge tubes, and frozen in aliquots at −80 °C until analyzed.

Pepstatin treatment was conducted by adding to the media (post-transfection) pepstatin A to a concentration of 100 μg/ml and MeSO to 2%. Control cultures were treated with 2% MeSO alone. Transfections and media collections were otherwise identical to untreated 293T cells.

**sAPP Competitive ELISA—** To generate and purify recombinant human sAPP, a 6-histidine tag was placed in-frame near the amino terminus of human APP695 by cleavage with KpnI and in-frame insertion of a double-stranded DNA fragment by annealing two oligonucleotides. After construction in the expression vector pAG3, the APP695ntermHis was stably transfected into CHO cells. For purification, after 48 h serum-free media was collected and purified using Ni2⁺ affinity resin (Qiagen). To perform sAPP96-well immunoassays, MaxiSorp 96-well immunoplates (Nunc) were coated overnight at 4 °C with 400 ng/ml purified recombinant sAPP in 100 μl of 0.1 M sodium carbonate buffer per well (pH 9.6). Plates were then blocked overnight at 4 °C with 1% Block Ace, 0.05% Tween 20, twice with PBS, and then loaded with 100 μl of TMB solution (Kirkegaard & Perry). The reaction was stopped after approximately 5 min with the addition of 100 μl of 6% phosphoric acid and the plates read at 450 nm. These conditions were determined empirically to optimize detection of sAPP in transfected cell lines in our laboratory, with typical expression levels placing sAPP concentrations in a linear region of the standard curve.

**Aβ Sandwich ELISAs and Normalization of Aβ Measurements—** For determination of Aβ concentrations we used 3 well characterized sandwich ELISA systems. Total Aβ was determined by 3160 capture and detection with either 4G8 or BNT77 (9, 26), and Aβ40 and 42 were determined by BAN50 capture and detection with either BA27 or BC05, respectively (5). To control for variance in transfection efficiency and the amount of APP that is appropriately inserted in the membrane and trafficked through the secretory pathway (thus APP available for γ-secretase cleavage) Aβ levels were normalized to sAPP expression. This was accomplished by dividing the Aβ values (fmol/ml) by the sAPP values (ng/ml) resulting in a normalized Aβ value (fmol/ng). The normalized Aβ value for each mutant was then divided by the normalized Aβ value for APP695NL to give the %NL Aβ.
Metabolic Labeling and Immunoprecipitation of sAPP and Full-length APP—48 h after transfection, 293T cells were labeled for 2 h with 100 μCi/ml [35S]methionine. Immunoprecipitation of full-length APP (tAPP) with Ab369W (27) from Triton X-100 cell lysates and sAPP in the 2-h conditioned media with antibody 207 were carried out as described previously (28, 29). Each analysis was performed in duplicate. Immunoprecipitated proteins were separated on 10% Tris-Tricine gels. PhosphorImaging analysis of the dried gels was performed. After subtracting the number of pixels of tAPP or sAPP present in the mock transfected (vector alone) sample from each of the experimental samples, the ratio of sAPP/tAPP was calculated by dividing the pixels of sAPP by the pixels of tAPP. sAPP/tAPP ratios were then compared with the ratio of sAPP/tAPP calculated for APP695NL. TMD mutants showed sAPP/tAPP ratios greater than 50% of APP695NL ratio were assumed to have preserved processing in the secretory pathway.

Mass Spectrometric Aβ Analysis—Serum-free media (Dulbecco’s modified Eagle’s medium) collected for a 24-h period beginning 24 h post-transfection was used for mass spectrometric analysis. Aβ peptides were identified by immunoprecipitation/mass spectrometric Aβ assay as described previously (4). The Aβ peptides were immunoprecipitated from 1.0 ml of conditioned media using monoclonal anti-Aβ antibody, 4G8 (Senetek, Maryland Heights, MO), and protein G Plus/Protein 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 Workstation, PerSeptive Biosystem). Each mass spectrum was averaged from 500 measurements and calibrated using bovine insulin as the internal mass calibrant. For comparing the peptide levels secreted by cells expressing different mutations and between the treatments of Me2SO and pepstatin, synthetic Aβ (12–28) peptide (10 nM) was used as internal standard.

RESULTS

Transmembrane Domain Mutations: Effects on Total Aβ Levels—APP appears to be a substrate for γ-secretase only after it has been cleaved by α- or β-secretase (30). Because cleavage of APP by these activities requires normal membrane insertion and trafficking through the secretory pathway, we have only performed in depth analyses of APP TMD mutants that showed preserved processing by these activities and normalized Aβ production to sAPP levels. Processing in the secretory pathway was assessed by metabolic labeling experiments and determination of the sAPP/tAPP ratio in comparison to APP695NL as described under “Experimental Procedures” (data not shown).

To examine Aβ production in the mutants that showed preserved processing in the secretory pathway, sAPP, total Aβ, Aβ1–40, and Aβ1–42 were measured in the conditioned media by ELISA. Comparison of normalized total Aβ shows that most mutants resulted in modest to moderate decrements in Aβ production (Table I). Even strikingly non-conservative mutations at residues 637 (Aβ1) or 639 (Aβ43) had relatively modest effects on total Aβ production. Basic (Lys) or acidic (Glu) amino acid substitutions at both sites, and substitutions of amino acids with a large hydrophobic side chain (Phe and Trp) or even a proline at position 637, did not prevent γ-secretase cleavage. In addition, production of Aβ1 was only modestly decreased by most mutations at or near the lumenal transmembrane domain junction or by deletion or insertion mutants. The notable exceptions being the large decrements in Aβ1 production by del640–43, 624–626E, and ins625–631 mutants. Six mutants (I637R, T639P, 649–651E, 649–651D, del625–631) showed dramatic decreases in Aβ1 levels (<5% of APP695NL Aβ, data not shown). Pulse-chase analyses showed that these mutations produced equivalent levels of full-length APP, but that the sAPP/tAPP ratio was markedly diminished (<1% compared with APP695NL, data not shown).

Although the precise mechanism for the impaired processing of these mutations is not definitively established by these studies, based on previous studies (16) and our metabolic labeling data it is likely that these mutants either alter membrane insertion or prevent proper trafficking through the secretory pathway. In agreement with this, one of these mutations (649–651E) has previously been shown to result in a non-transmembrane, membrane-associated cell surface. full-length APP (31).

Effects on Cleavage Site—Comparison of the relative amounts of Aβ1–40 to Aβ1–42, measured, respectively, by
γ-Secretase Cleavage of Aβ

Results of total Aβ ELISAs, standardized to sAPP levels (assayed in duplicate, mock transfection levels subtracted). Values shown are the average of three to five independent transfections for each mutation; control levels for APP695NL are determined from 17 transfections to ensure the most accurate estimate possible. %Aβ1–40 and %Aβ1–42 are expressed as mean ± SD. Statistical significance (*, p < 0.05; **, p < 0.01; ***, p < 0.001) was assessed by computing values from Student’s t distribution by comparing each mutation to the control population of APP695NL transfections.

| Construct | %NL Aβ* | %Aβ1–40 | %Aβ1–42 | Major effects |
|-----------|---------|---------|---------|--------------|
| I637W     | 49***   | 6***    | 1.0***  | ↓Aβ40, ↓Aβ42, ↑Aβ7** |
| I637P     | 60*     | 1***    | 0.0***  | ↓Aβ40, ↓Aβ42, ↑Aβ7? |
| I637F     | 73*     | 34      | 1.0***  | ↓Aβ40, ↓Aβ42, ↑Aβ8 |
| I637E     | 28***   | 8***    | 0.2***  | ↓Aβ40, ↓Aβ42, ↑Aβ7 |
| I637K     | 85      | 52      | 2.6     | Slight ↓Aβ42 |
| T639K     | 55**    | 41      | 1.0***  | ↓Aβ42 |
| T639E     | 59**    | 45      | 3.1     | |
| T639A     | 39***   | 33      | 9.7***  | ↓Aβ42 |
| 640–648A  | 69**    | 28*     | 4.4     | ↓Aβ40, ↑Aβ2 |
| 625–626K  | 94      | 14***   | 11.5*** | ↓Aβ40, ↑Aβ42, ↑Aβ7 |
| G625P     | 78      | 30*     | 3.2     | ↓Aβ40, ↑Aβ2 |
| 624–626D  | 39***   | 22*     | 54.0*** | ↓Aβ40, ↓Aβ42 |
| 624–626E  | 9***    | 25*     | 8.7***  | ↓Aβ40, ↓Aβ42, ↑Aβ7 |
| del625–628| 62**    | 14***   | 48.1*** | ↓Aβ40, ↑Aβ42 |
| ins625–628| 27***   | 2***    | 4.6     | ↓Aβ40, ↑Aβ7 |
| ins625–631| 18***   | 6***    | 1.9     | ↓Aβ40, ↑Aβ7 |
| del640–643| 18***   | 69      | 40.5*** | ↓Aβ42, ↑Aβ7 |
| del644–647| 71*     | 36      | 1.8*    | ↓Aβ42 |
| ins644–647| 81      | 6***    | 4.7*    | ↓Aβ40, ↑Aβ42, ↑Aβ7 |
| APP695NL  | 100     | 51 ± 4.2| 3.3 ± 0.3| |

* Aβ7, Aβ species other than those ending at 40 or 42.

BAN50/BA27 or BAN50/BC05 ELISA, shows that many of these mutants dramatically alter the relative amounts of the major Aβ species produced. These data are expressed as the percent of total Aβ that Aβ1–40 and Aβ1–42 species represent (Table I). For the I637X mutants, detection of Aβ1–42(43) species could be impaired as the 42(43) end specific BC05 detection antibody recognizes an epitope partially determined by the residue at Aβ41; thus, altered Aβ42 peptides could be produced but not detected (5). To illustrate how these data indicate shifts in cleavage it is useful to look at the effects on %Aβ1–40 and %Aβ1–42 in the ins625–628 mutant. Comparison of the %Aβ1–40 and %Aβ1–42 illustrates that the major shift in cleavage is a reduction in processing at the Aβ40 cleavage site (2% of total versus 51% of total in the APP695NL construct) while Aβ42 cleavage is preserved (4.6% of total versus 3.5% of total in the APP695NL construct). These analyses also show that only 7% of total Aβ is accounted for by Aβ1–40 and 1–42 versus 54% in the APP695NL construct, indicating that the vast majority of Aβ peptides generated from this mutant are cleaved at a different site or sites than the ones normally utilized. By these criteria, almost all of the mutants show shifts in γ-secretase cleavage. In the last column of Table I, the statistically significant shifts in cleavage for each of these mutations are summarized.

Mass Spectral Analysis—In order to determine exactly how cleavage is shifted by the TMD mutations, Aβ produced from 293T cells expressing APP695NL or select TMD mutant APPs was analyzed by immunoprecipitation/mass spectrometric analysis (4). The monoclonal antibody, 4G8, was used to immunoprecipitate Aβ from serum-free conditioned media. This antibody recognizes Aβ17–24 (32), a region of the Aβ not altered by the mutants used in this study, making it highly unlikely that this analysis would be biased by selective immunoprecipitation of different Aβ species. The molecular masses of various Aβ peptides were measured in these analyses by using internal mass calibrants, bovine insulin and Aβ12–28 peptide. These masses were then used to identify Aβ peptides produced by the TMD mutant APPs and infer the γ-secretase cleavage sites as illustrated in Fig. 3. The relative peak intensity was used to determine the relative abundance of Aβ peptides within each spectra resulting from each TMD mutant APP. For clarity, all Aβ species were numbered according to the cleavage sites in wild type Aβ species; thus Aβ1–43 in del625–628 is a 39-amino acid Aβ peptide derivative. Representative mass spectra for APP695NL, 1637F, 1637P, and ins644–647 are shown in Fig. 3A.

The mass spectrum of Aβ produced from APP695NL shows that Aβ1–40 was the major Aβ species and that the minor Aβ species were Aβ1–42, Aβ1–39, Aβ1–38, and Aβ1–37. Based on comparison to APP695NL, several of the different TMD mutations analyzed dramatically shift the γ-secretase cleavage site. Mass spectrometric Aβ analyses of APP695NL and TMD mutants are schematically summarized in Fig. 3B. The largest shifts in cleavage site utilization are seen in the ins625–628 and del625–628 mutants, while less dramatic effects are seen with the del644–647 and ins644–647 mutations. G625K also increases long Aβ production, while G625P increases production of shorter Aβ peptides. Introduction of Lys at position 637 or 639 has only minor effects on cleavage, while substitution of Ala at 639 increases both long Aβ (Aβ1–42) and short Aβ (Aβ1–38) production. Pro or Phe substitutions at Ile637 dramatically shift cleavage away from Aβ40. Finally, Aβ production is only minimally affected by the 640–648A mutant. Notably, the mass spectral data and the ELISA data are remarkably consistent with the exception being that small amounts of Aβ1–42 detected by ELISA are not always detected by mass spectrometric analysis (for example, in 640–648A) due to the slightly lower detection efficiency of Aβ1–42 (4).

Pepstatin Treatment of Selected Mutants—Given the loose sequence specificity exhibited by γ-secretase, we postulated
that the γ-secretase activity responsible for generating the various Aβ species was unlikely to be due to a single protease. Therefore, we treated cells transfected with several of the TMD mutants that shift cleavage with pepstatin, an inhibitor of normal γ-secretase activity. Although it is not known whether pepstatin directly inhibits γ-secretase, pepstatin treatment of cells transfected with APP COOH-terminal fragments LC99, APP695wt, or APP695NL results both in accumulation of COOH-terminal fragments and a decrease in Aβ production\(^2\) without significantly altering sAPP secretion in either APP695wt or APP695NL (Table II), consistent with it being an inhibitor of γ-secretase activity. Because cells are rather impermeable to pepstatin, it is necessary to treat them with high

\(^2\) C. Eckman, unpublished results.
concentrations (100 μg/ml) in the presence of 2% Me₂SO in order to obtain effective intracellular concentrations (33). Again sAPP, total Aβ, and Aβ1–40 and 1–42 were measured by ELISA from conditioned media. Treatment of cells with vehicle alone and vehicle with pepstatin had no overt toxic effect in the time course studied, and although changes in sAPP levels were observed in individual experiments in some mutants, none of these changes reached statistical significance (Table II). Pepstatin treatment of cells transfected with the APP695NL mutant decreased total Aβ and Aβ1–40 equally while Aβ1–42 was less affected. The effects on three of the TMD domain mutants: del625–628, G625P, and I637F were similar to the effect on APP695NL. In contrast, pepstatin treatment did not alter Aβ production from the I637P mutant to any significant extent. The effects of pepstatin on T639K, a mutant that had only subtle effects on cleavage site utilization, were also distinct from APP695NL. Aβ1–40 was only modestly inhibited and Aβ1–42 was not inhibited. Although Aβ1–40 produced from ins625–628 represents only a minor fraction of the total Aβ produced, pepstatin actually increased the amount of Aβ1–40 produced from 15 to 50 fmol/ml.

To definitively identify how pepstatin altered cleavage of these TMD domain mutants, Aβ secreted from treated and untreated cells was analyzed by mass spectrometry (Fig. 4). For APP695NL, G625P, I637P, and T639K, the relative peak heights of each of the major Aβ species was similar before and after pepstatin treatment, indicating that cleavage was inhibited equally at each site (Fig. 4, data shown only for APP695NL). Consistent with the ELISA analysis, the relative peak height of Aβ1–42 was not reduced as much by pepstatin treatment in APP695NL and G625P. The effect of pepstatin treatment on del625–628 also revealed little difference in relative peak heights of the major Aβ species except that peaks corresponding to Aβ1–45 and Aβ1–46 were increased suggesting enhanced utilization of minor cleavage sites (Fig. 4, G and H). In the ins625–628 and I637P TMD mutants, there were some marked shifts in the relative peak intensities of some Aβ species after pepstatin treatment. In ins625–628 the Aβ1–33 peak was decreased more than the Aβ1–37 peak (Fig. 4, E and F), while in I637P the relative peak intensity of Aβ1–37 decreased and the relative peak intensity of Aβ1–43 increased (Fig. 4, C and D). Thus, there appears to be differential sensitivity of certain γ-secretase cleavages to pepstatin and increased utilization of alternative sites by pepstatin-insensitive proteases in certain mutants when pepstatin-sensitive cleavage is inhibited.

### DISCUSSION

**APP Transmembrane Domain Mutants Shift γ-Secretase Cleavage**—An unanticipated result of this analysis is that many TMD mutations alter the major sites of γ-secretase cleavage. The most striking finding resulting from the analyses of the mutations, ins625–628 and del625–628, is that the major γ-secretase cleavage site appears to be determined by the length of the TMD luminal to the normal γ-secretase sites. In wild type APP, the major γ-secretase site carboxyl to Aβ40 lies 12 amino acids from the lysine at Aβ28 (KA28) that is predicted to delineate the luminal TMD boundary. In these mutations, the normal γ-site is shifted either 4 amino acids (del625–628) closer or farther away (ins625–628) relative to KA28. Although the primary sequence surrounding the normal γ-site is unaltered, the major cleavage shifts to the 13th amino acid (Aβ37) from KA28 in ins625–628 or the 11th amino acid (Aβ43) from KA28 in del625–628. Only a minor portion of Aβ produced from either mutant is cleaved at the V-I bond at Aβ40 (14% in del625–628 and 2% in ins625–628). Mutations altering charge at the presumptive luminal border of the transmembrane domain (625–626K, 624–626E, and 624–626D) had a similar effect as the del625–628 mutant increasing production of longer Aβ peptides. These mutants might be predicted to decrease the length of the TMD proximal to the γ-secretase site by several amino acids resulting in increased cleavage at sites distal to Aβ40.

The insertion and deletion mutants designed to alter the length of the TMD distal to the normal γ-cleavage sites had much subtler effects. The major effect of the ins644–647 mutation was to decrease cleavage at Aβ40 and increase cleavage at Aβ38. The major effect of the del644–647 mutant was to modestly decrease Aβ1–42 production. This is similar to the effect seen in the I637K and T639K mutants, where Aβ1–42 production is decreased. In all three cases, increased positive charge has been placed closer to the normal Aβ42 cleavage site. Replacement of residues 640–648 with alanine (640–648A) had only a minor effect on Aβ production shifting cleavage away from Aβ40 site (28% Aβ40–41) indicating that this region is not as crucial in determining the membrane positioning of the γ-secretase sites in comparison to the region amino to the γ-secretase cleavage site. This finding is similar to a previous report showing that substitution of APP residues 635–642 or 636–653 with the corresponding residues of the epidermal growth factor receptor TMD did not significantly impair Aβ production (16). Nevertheless, alterations in this region do result in subtle, but important, shifts of γ-cleavage. All FAD-linked mutations in this region, V641I, V641F, V641G, and I640V (based on the APP695 sequence), replace hydrophobic residues in the transmembrane domain with other hydrophobic residues and each results in increases in Aβ42 (5, 17, 34; reviewed in Refs. 7 and 8). In this study, a similar substitution, T639A, increased Aβ42 cleavage. This result is similar to a recent report demonstrating that hydrophobic substitutions at Aβ43 increased Aβ42 production relative to Aβ40 (17). However, that study only looked at ratios of Aβ42:Aβ40; thus, alternative cleavages would have been missed, and it is unclear from the data presented whether the increase in the ratio is due to a decrease in Aβ40 production or an increase in Aβ42 production. Of the mutations on the carboxyl side of the γ-site, only del640–643 had dramatic effects on Aβ, significantly decreasing total Aβ production and markedly increasing %Aβ1–42 production. This mutant’s more dramatic effect compared with deletions or insertions downstream could be explained by the fact that this region may represent the end of the APP TMD (16). Taken together, these data indicate that mutations in the luminal portion of the TMD have effects on

### Table II

**ELISA analysis of pepstatin treatment on select TMD mutants**

| Construct | sAPP | Total Aβ | Aβ 1–40 | Aβ 1–42 |
|-----------|------|----------|---------|---------|
| I637P     | 114% | 10%      | ND      | ND*     |
| DEL625–628| 91%  | 32%**    | 32%**   | 44%*    |
| G625P     | 12%  | 17%**    | 21%**   | 40%*    |
| I637F     | 15%  | 26%**    | 27%**   | 54%     |
| T639K     | 69%  | 57%      | 72%     | 123%    |
| INS625–628| 115% | 59%**    | 34%**   | ND      |
| NL        | 115% | 17%**    | 22%**   | 34%*    |

* ND, indicates these species were not detected by ELISA in these experiments.
cleavage that are much more profound than mutations which alter charge or TMD length on the cytoplasmic side of the normal γ-secretase site.

γ-Secretase Is Not a Single Proteolytic Activity—Our data shed some light on the proteolytic mechanism responsible for generation of Aβ peptides of various lengths. The finding that the del625–628 preferentially secretes an Aβ species of 39 amino acids ending at Aβ43 is evidence against a mechanism in which a single endoprotease cleavage occurs carboxyl to the major γ-sites followed by trimming by carboxypeptidase. However, for any given mutation, we cannot rule out the possibility that shorter Aβ species are generated through carboxypeptidase degradation of longer Aβ peptides. Furthermore, the differential effect of pepstatin on select TMD mutants is most consistent with the action of at least two and possibly more proteolytic activities. This differential inhibition most clearly reflects a varying degree of organelle penetrance (a similar effect has previously been shown to be specifically generated in the endoplasmic reticulum (although endoplasmic reticulum-derived Aβ1–42 is not secreted), it is possible that this difference simply reflects a varying degree of organelle penetrance (a similar effect could be made for any of the other published inhibitors) (40–42). Alternatively, this differential inhibition could be viewed as additional evidence that multiple proteases generate both Aβ40 and Aβ42, but that pepstatin-sensitive cleavage is responsible for a higher percentage of activity at the Aβ40 site. The finding that differential inhibition of Aβ1–40 and Aβ1–42 was constant among several of the TMD mutants which alter cleavage site preference (del625–628, G625P, I637F), but not the T639K mutant, supports this notion of multiple proteases as it is most consistent with enhancing the Aβ40 and Aβ42 cleavages by a pepstatin-insensitive protease. Based on our results, we would predict that for wild type

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**FIG. 4. Mass spectral analysis of Aβ secreted by TMD mutants in the presence or absence of pepstatin.** Immunoprecipitation/MS spectra of Aβ peptides secreted by 293T cells expressing APP695NL (A and B), 1637P (C and D), ins625–628 (E and F), and del625–628 (G and H) proteins after treatments with either MeSO (vehicle) (A, C, E, and G) or pepstatin (B, D, F, and H). For each pair of spectra, MeSO versus pepstatin, the peak heights of Aβ peptides are first normalized to the internal standard, Aβ12–28 (labeled as 12–28 (Std)). The spectra are then plotted using the relative peak intensities; e.g. the highest peak within the pair of spectra is plotted as 100%. In this way, the relative intensity of peaks between the two spectra can be compared. Peaks in the spectra are labeled with Aβ peptide sequence numbers based on the wild type Aβ sequence. Thus, Aβ1–37 in ins625–628 is a 41-amino acid peptide whereas Aβ1–43 in del625–628 is a 39-amino acid peptide, respectively. Background peaks and unidentified peaks are labeled with asterisk (*) and question marks (?), respectively.

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of these previous studies has been difficult. The compound, MDL 21760, used in several studies could have altered trafficking of APP as it markedly increased sAPP (35, 36). In other studies, reporting an Aβ1–40 inhibitory effect of calpain 1 inhibitor, no controls for either APP synthesis or sAPP production were reported (37, 38). In fact, only one potential γ-secretase inhibitor, a substrate-based difluoroketone compound, altered γ-secretase activity without apparent alteration of other secretase activity (39). In this study, pepstatin had no toxic effects and no significant effect on sAPP production from APP695NL; yet, it was a less effective inhibitor of Aβ1–42 (~35% of control) production than Aβ1–40 (~20% control).

The differential inhibition observed at Aβ40 and Aβ42 sites could be due to a number of mechanisms. Since Aβ1–42 has previously been shown to be specifically generated in the endoplasmic reticulum (although endoplasmic reticulum-derived Aβ1–42 is not secreted), it is possible that this difference simply reflects a varying degree of organelle penetrance (a similar argument could be made for any of the other published inhibitors) (40–42). Alternatively, this differential inhibition could be viewed as additional evidence that multiple proteases generate both Aβ40 and Aβ42, but that pepstatin-sensitive cleavage is responsible for a higher percentage of activity at the Aβ40 site. The finding that differential inhibition of Aβ1–40 and Aβ1–42 was constant among several of the TMD mutants which alter cleavage site preference (del625–628, G625P, I637F), but not the T639K mutant, supports this notion of multiple proteases as it is most consistent with enhancing the Aβ40 and Aβ42 cleavages by a pepstatin-insensitive protease.
substrate a pepstatin-sensitive protease cleaves both Aβ40 and Aβ42, with preferential specificity for cleavage at Aβ40 and that additional pepstatin-insensitive proteases account for ~20% of the cleavage events at Aβ40 and ~35% of the cleavage events at Aβ42. Thus, when the major Aβ40 cleavage site is “protected” (see below) from cleavage in the del625–628 mutant (14% Aβ1–40 versus 51% in APP695NL, Table II) and shifted to Aβ1–42(43) (48% versus 3.3% in APP695NL, Table II), a remarkably similar degree of inhibition is seen on both species following pepstatin treatment. A final mechanism in which a single pepstatin-sensitive protease cleaves both sites but is differentially inhibited could also be considered. This mechanism, however, is not consistent with known models of proteolytic inhibition by pepstatin and would be an unprecedented mechanism of action for a protease inhibitor that acts in a competitive fashion.

**Implications for Models of γ-Secretase Cleavage: the Length of the Lumenal Portion of the TMD Is the Prime Determinant of Cleavage**—Until the γ-secretases are definitively identified and the location of the residues at the time of cleavage defined, the issue of intramembranous proteolysis is likely to remain controversial. Unless γ-secretases are completely novel proteases that can cleave proteins in the hydrophobic environment of the membrane, mechanisms must exist that permit membrane-embedded regions of polypeptides to transfer from the lipid bilayer into a hydrophilic proteinaceous environment that supports proteolysis. This could occur in one of two fashions. First, as has been generally proposed, γ-secretase activity may be due to the action of proteases or proteolytic complexes that are capable of creating a pore or hydrophilic pocket within the membrane. Based on our data, in this model the interaction of the protease with the TMD domain of the APP lumenal to the γ-secretase site would appear to be a critical factor in determining cleavage (see Fig. 5A). A second possibility is that these cleavages require translocation of the cleavage sites out of the membrane (Fig. 5B). One of the similarities between the γ-secretase-like cleavages in APP, Notch and SREBP, is that the potentially intramembranous cleavage occurs only after an initial cleavage or cleavages that remove significant portions of the protein amino-terminal to the intramembranous cleavage site. Thus, it is possible that the sites are normally protected by the membrane prior to the primary lumenal cleavage of the holoprotein. Once the lumenal domain is cleaved, the protein either assumes a different conformation or is actively translocated resulting in exposure of the intramembranous site to the cytoplasm where it can be cleaved. Such a cut-expose-cut model for the APP is illustrated in Fig. 5B. In this model one could envision slippage or translocation of the intramembranous site in either direction; for APP we propose that the γ-site is exposed to proteases that are associated directly or indirectly with the membrane and have active sites facing the cytoplasm. This model, while subject to some potential thermodynamic penalties associated with translocation of the APP TMD, is consistent with our data showing that length of the TMD lumenal to the γ-cleavage site has more profound effects on γ-secretase cleavage than mutations carboxyl to the normal γ-sites, offers a very simple explanation as to why these sites are resistant to proteolysis in the intact holoprotein, is consistent with another model of intramembranous cleavage (21), and does not imply an unprecedented type of proteases in this cleavage.

In either model proposed above, it is apparent that the prime determinant of γ-secretase cleavage is the length of the transmembrane domain proximal to the γ-secretase cleavage site. In an intramembranous cleavage model, we would predict that the position of the active site of the protease is relatively fixed within the membrane and that the protease recognizes determinants in the APP TMD lumenal to the normal cleavage site. Thus, varying the length of the lumenal TMD alters the residues that contact the active site. In contrast, the membrane serves as the delineating factor in the cut-expose-cut model, residues buried within the membrane are protected from cleavage while exposed residues are cleaved.

Because at least two, and possibly more, proteolytic activities appear to contribute to γ-secretase cleavage, defining the substrate specificity is problematic until the secretases are definitively identified. Nevertheless, it is almost certain that any individual γ-secretase would exhibit at least some sequence specificity as even nonspecific proteases such as proteinase K preferentially cleave certain substrates (43, 44). Consistent with this, altering sequence at the normal γ-cleavage site has dramatic effects on recognition of the normal cleavage site by the major pepstatin-sensitive γ-secretase activity which results in either increased cleavage by a pepstatin-sensitive protease at sites other than Aβ40 and Aβ42, increased cleavage by a pepstatin-insensitive protease at normally utilized sites, increased cleavage by a pepstatin insensitive protease at Aβ40 and Aβ42, or a combination of these alterations.

**Multiple Cellular Factors Could Influence γ-Secretase Cleavage**—If γ-secretase cleavage is primarily dependent upon the location of the γ-secretase cleavage site with respect to the membrane or active site of a protease within the membrane, then it is likely that a number of cellular factors could influence this cleavage including membrane thickness or composition and interaction with other proteins. PSs, which are important regulators of γ-secretase activity (reviewed in Refs. 7 and 8), could influence γ-secretase cleavage of APP by altering trafficking of APP to different cellular microdomains where cleavage would be influenced by membrane thickness, by directly interacting with APP carboxyl-terminal fragments and positioning them within the membrane, by translocating the γ-site out of the membrane, or by altering the position of γ-secretase with respect to the APP transmembrane domain. Alternatively, the possibility that PSs, which do not resemble any known protease, could in fact be γ-secretase has not been excluded, in which case altered interaction between PS and APP could provide a simple explanation for shifts in cleavage induced by FAD-linked PS mutants and would be entirely consistent with decreased production of Aβ from PS 1 knockout mice (45).

**Conclusions**—γ-Secretase appears to represent a membrane protein secretase defined as a proteolytic activity that cleaves a proteolytically sensitive region of a transmembrane protein resulting in secretion of the proximal portion of the cleaved protein (46). Compared with other secretase activities, γ-secretase activity appears to be distinct in that it does not cleave its substrate within an extracellular/lumenal “stalk” like region proximal to the membrane, but rather within a hydrophobic region purportedly within the TMD of the APP COOH-terminal fragment. The demonstration in this study that the γ-secretase is not a single proteolytic activity and that the membrane plays a critical role in determining γ-secretase cleavage of APP will be important in developing strategies for isolation of the various γ-secretase activities, which remain a major therapeutic target in AD. Additional studies will be needed to determine whether the pathological shifts in Aβ cleavage are caused by the alterations in the major pepstatin-sensitive γ-secretase activities or by additional proteases, which might play a role in pathogenic processing. Given that the common effect of all early onset FAD-linked mutations is to modestly increase long Aβ production, this study offers important insight into how various Aβ peptides are generated and suggests possible mechanisms whereby various FAD-linked mutations might shift...
A. Intramembranous Cleavage

β-Secretase

Lumen/Extracellular

DAEF..........AEDVGSNKGAIGLMVGGVIATVITTLVMLKKQYTS......

β-Secretase

Cytoplasm

DAEF..........AEDVGSNKGAIGLMVGGVIATVITTLVMLKKQYTS......

Aβ40

wt

del625-628

ins625-628

B. Cut-Expose-Cut

β-Secretase

Lumen/Extracellular

DAEF..........AEDVGSNKGAIGLMVGGVIATVITTLVMLKKQYTS......

β-Secretase

Cytoplasm

DAEF..........AEDVGSNKGAIGLMVGGVIATVITTLVMLKKQYTS......

Aβ40

wt

del625-628

ins625-628

FIG. 5. Models of γ-secretase cleavage. This figure illustrates two potential models for γ-secretase cleavage. A, intramembranous cleavage. In this model, the APP COOH-terminal fragment generated after β-secretase cleavage associates with a protease whose active site lies within the membrane. Based on our data, the interaction of the intramembranous protease with the TMD domain of the APP luminal to the γ-secretase site would appear to be a critical factor in determining cleavage. Thus, del625–628 and ins625–628 mutants would alter the residues in contact with the active site resulting in different cleavages. B, a cut-expose-cut model. In this model, prior to cleavage by α- or β-secretase, the γ-site is buried within the membrane, inaccessible to proteolysis. After cleavage to release sAPP, the APP COOH-terminal fragment location within the membrane is altered to expose the γ-site to the cytoplasm where it could be cleaved by either cytoplasmic proteases or membrane-associated proteases with active sites facing the cytoplasm. This translocation could occur because this is the preferential conformation of the APP COOH-terminal fragment within the membrane or it could be facilitated by interaction with PS, γ-secretase itself, or other transmembrane proteins. Such a model provides a simple explanation for the cleavages observed in the del625–628 and ins625–628 mutant APPs. Based on this model, thickness of the membrane could influence cleavage site or alternatively interaction with other proteins in the membrane, such as PS, could alter the exposure of the γ-site. Because the transmembrane region of a protein is not static within the membrane, it is likely that in either model there is some resonance of the COOH-terminal fragment with respect to the membrane. Such resonance could result in either different residues presented to the active site of an intramembranous protease or exposure of different residues to the cytoplasm, which could account for the invariable production of “ragged” ends. Scissors indicate proteolytic events. β and γ indicate β- and γ-secretase cleavage, respectively.

γ-secretase cleavage. Because other biologically important cleavage events in SREBP and Notch may be intramembranous, it will be important to perform similar studies on such proteins to determine if similar, atypical, proteolytic mechanisms are responsible for such cleavages. Together, these studies should also offer additional insights into the important question of how transmembrane domains, in general, are degraded.

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