

Influence of the familial Alzheimer's disease-associated T43I mutation on the transmembrane structure and γ -secretase processing of the C99 peptide

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Running Title: *Structure of the T43I mutant of C99*

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ABSTRACT

Extracellular deposition of amyloid- β (A β) peptides in the brain is a hallmark of Alzheimer's disease (AD). Upon β -secretase-mediated cleavage of the β C-terminal fragment (β -CTF) from the A β precursor protein, the γ -secretase complex produces the amyloid β (A β) peptides associated with AD. The familial T43I mutation within the transmembrane domain of the β -CTF (also referred to as C99) increases the ratio between the A β 42 and A β 40 peptides largely due to a decrease in A β 40 formation. A β 42 is the principal component of amyloid deposits within the brain parenchyma, and an increase in the A β 42:A β 40 ratio is correlated with early-onset AD. Using NMR and FTIR spectroscopy, here we addressed how the T43I substitution influences the structure of C55, the minimal sequence containing the entire extracellular and transmembrane (TM) domains of C99 needed for γ -secretase processing. ^{13}C NMR chemical shifts indicated that the T43I substitution increases helical structure within the TM domain of C55. These structural changes were associated with a shift of the C55 dimer to the monomer and an increase in the tilt of the TM helix relative to the membrane normal in the T43I mutant compared with that of wild-type C55. The A21G (Flemish) mutation was previously found to increase secreted A β 40 levels; here, we combined this mutation in the extracellular domain of C99 with T43I and observed that the T43I/A21G double mutant decreases A β 40 formation. We discuss how the observed structural changes in the T43I mutant may decrease A β 40 formation and increase the A β 42:A β 40 ratio.

The extracellular deposition of amyloid- β (A β) peptides in the brain is a prominent feature of Alzheimer's disease (AD) and is caused by proteolysis of the amyloid precursor protein (APP) via a sequential two-step process. Cleavage of APP by β -secretase near the boundary between ectodomain and extracellular juxtamembrane (JM) sequence causes shedding of the ectodomain, and is required for a second cleavage within the single transmembrane (TM) helix of APP (1). The γ -secretase complex catalyzes the second cleavage, which leads to the formation of the A β peptides with lengths ranging from 38 to 42 amino acids.

A β 40 is the most prevalent A β peptide. However, A β 42 has a higher propensity to form aggregates than the shorter isoforms and is the most toxic peptide generated by γ -cleavage (2). A β 42 is also the principal component of amyloid plaques in AD patients (3).

Familial AD mutations occur in both the γ -secretase complex and in the APP substrate. The first mutations identified in APP were at Ala21, Glu22 and Asp23 within a cluster in the extracellular region of the protein. (In the following, we use the β -CTF numbering, which also coincides with the numbering of the A β peptides, i.e. Asp1 is the first residue of the β -CTF and the A β peptide.) These mutations included the A21G Flemish, E22Q Dutch, E22G Arctic, E22K Italian, and D23N Iowa mutations (4-7). Within this cluster, mutations have been found to influence different stages of the conversion of APP to amyloid fibrils. For example, the A21G mutation increases the total amount of secreted A β by enhancing γ -secretase processing of the β -CTF (8,9). In contrast, mutations at Glu22 and Asp23 increase the rate of fibril formation following the formation of the A β peptides (10), but do not influence proteolysis by γ -secretase (9). Over the past few years the mechanisms by which these mutations influence the proteolysis of APP or the conversion of A β monomers to fibrils have begun to emerge (11,12).

A second cluster of amino acids in the β -CTF that are mutated in familial AD occurs between the cleavage site (Ala42) that releases the A β 42 peptide and the cytoplasmic end of the TM domain (Leu52). These include the T43A Iranian (13), T43I Austrian (14), V44M French (15), V44A German (16), I45V Florida (17), V46I London (18), V46L Indiana (19), V46G (20) and L52P Australian (21) mutations. Gorman *et al.* (22) measured the influence of three FAD mutations (T43I, V46G and V46F) on the monomer-dimer equilibrium in the context of TM peptides. They found a correlation between the dissociation constant for the TM peptide dimer and the A β 42/40 ratio observed in AD patients, suggesting that processing of the APP monomer results in an increase in A β 42. More recent studies indicated that mutations in the four-residue stretch from Thr43 to Val46 decreased homodimerization and

increased the A β 42/40 ratio in the context of the β -CTF (11). However, it is not clear how four sequential residues in the C99 sequence can decrease homodimerization since only two residues at most might lie in the dimer interface.

Here, we focus on the Austrian T43I mutation, which is located in the TM cluster of mutations to establish the structural changes that occur upon mutation. Recent NMR studies suggest that the region near the γ -secretase cut site does not form a stable α -helix in the wild-type protein (23). One possibility is that it is unraveled to facilitate cleavage in the dimer or binding to the γ -secretase complex. It is known that the TM helix must unravel to undergo proteolysis. We propose that in the monomer, backbone hydrogen bonding interactions favor α -helix formation, which impede enzyme-substrate interactions, substrate unraveling and proteolysis.

T43I causes the largest increase in the A β 42/A β 40 ratio within the cluster of TM FAD mutations (14). Thr43 is one amino acid after the γ -cleavage site that generates the A β 42 peptide. This site is roughly midway between the extracellular and intracellular boundaries. On the extracellular side of Thr43, there are several GxxxG motifs that mediate dimerization of the TM domain in membrane bilayers (24) and have been implicated in cholesterol binding (25). On the intracellular side of Thr43, is a cluster of β -branched amino acids (Val44, I45, V46) whose mutation results in early onset AD.

To better understand the influence of the T43I mutation on γ -secretase processing, we also combine it in a single construct with the A21G mutation located in the extracellular cluster of FAD mutants. We have previously shown that the A21G mutation lengthens the TM helix, stabilizes the TM dimer and destabilizes β -sheet structure in the extracellular region of the β -CTF (8,12). These structural changes are associated with an increase in the total amount of A β peptide released upon γ -secretase cleavage, as well as with an increase in the A β 42/40 ratio (8,9). By combining an extracellular FAD mutation (A21G) and a TM FAD mutation (T43I), we can probe whether the two clusters of FAD mutations act independently or synergistically in γ -secretase processing.

We characterize the influence of the T43I and A21G mutations on the structure of C55, a 55-residue peptide that includes the extracellular and TM regions of C99. We have previously shown that the extracellular and TM structures are similar in C55 and C99, and that differences in the amounts of A β 38, A β 40 and A β 42 produced using wild-type or selected mutant sequences are similar (8). The similarity in processing likely stems from the fact that the intracellular domain of C99 is unstructured and removed in the initial ϵ -cleavage of C99, yielding a product that is the same as C55 after ϵ -cleavage.

Structural measurements using solution and solid-state NMR spectroscopy are carried out in detergent micelles and in membrane bilayers, respectively. Chemical shift measurements upstream and downstream of the point of mutation provide evidence for global changes in structure, while measurements of inter-helical dipolar couplings assess the dimerization state of wild-type C55, the T43I mutant and the A21G/T43I double mutant of C55. FTIR measurements also provide information on the global secondary structure of these mutants in membrane bilayers.

RESULTS

Mutations at Thr43 Change the Production and Ratio of A β 42 to A β 40—There are two mutations at Thr43 that have been described in the literature, the T43A Iranian (13) and T43I Austrian mutations (14). These introduce different size side chains at position 43, and both result in early onset AD. To better understand the dependence of amino acid type at position 43 on processing, we analyzed the distribution of secreted A β 38, A β 40 and A β 42 produced by the expression and processing of wild-type C99 and a series of Thr43 mutants in Chinese hamster ovary (CHO) cell cultures, which are widely used as they generally produce more A β peptides through γ -secretase processing than primary neurons.

The mutants within the TM of C99 selected for study comprise substitutions of Thr to hydrophobic or weakly polar amino acid residues (Figure 1). The amino acid substitutions chosen are conservative and transfected DNA constructs express at comparable levels in CHO cells. The observed FAD mutations in cluster 2 tend to all be

conservative and involve substitutions with different hydrophobic side chains. This observation suggests that non-conservative mutations prevent C99 processing. The overall production of A β peptides is generally decreased by substitution of Thr43. The familial AD Thr43 mutations, T43I and T43A, exhibit decreased total A β production (i.e. A β 38, A β 40, A β 42) by ~65 and 45%, respectively. Mutation of T43I and T43V dramatically decreases the level of A β 40 and increases A β 42. The A β 42/40 ratio is highest for T43I. These results are dramatically different from those of the A21G mutant and other sites in the adjacent LVFF sequence in which mutation leads to an increase in total A β production (8,9).

Serine and valine are perhaps the most similar amino acids to threonine. Both have roughly the same molecular volume as threonine. Serine has a β -hydroxyl group, while valine has a β -methyl group. The T43S mutation results in a similar loss of total A β production (~50%), as observed for T43I and T43A, but has almost no effect on the A β 42/40 ratio. The T43V mutation closely resembles T43I in total production and has the second highest increase in the A β 42/40 ratio. Together these results suggest that loss of the β -hydroxyl group of Thr43 has a crucial influence on the A β 42/40 ratio.

Of the amino acids tested, glycine and phenylalanine are structurally the most different from threonine. A β production is blocked by the T43F mutation, which places a large hydrophobic residue at position 43, whereas T43G exhibits a striking increase in A β 38 relative to A β 40 and A β 42.

Overall, the changes in γ -secretase processing are not correlated with the size or character of the amino acid at position 43, other than loss of the β -hydroxyl group. The T43F mutant exhibited the lowest amount of soluble A β 40 of the mutants tested, whereas T43M generated a level of A β 40 close to wild-type. It is interesting to note here that phenylalanine substitutions at V44, I45, V46, I47 and T48 have very different influences on total A β and the A β 42/40 ratio (26). One general correlation of the T43 mutants is a decrease in the total A β produced as observed previously for T43A and T43I (27). A

large decrease in A β 40 secretion is the primary cause of an increase in the A β 42/ A β 40 ratio for the T43I mutation.

Comparison of Wild-Type C55 and Thr43 Mutants Reveal Global Changes in Structure— C55 is the minimal sequence containing the entire extracellular and TM domains of C99 needed for γ -secretase processing (8). To assess the global structure of C55, we undertook solution NMR measurements in dodecylphosphocholine (DPC) detergent micelles. These studies parallel those in DPC micelles on the full β -CTF molecule (residues 1-99) (25,28), a truncated β -CTF (residues 15 – 53) (29), and TM domain alone (residues 28-55) (30). Figure 2 presents the 2D ^{15}N - ^1H HSQC spectrum of detergent solubilized C55. This construct (designated C55*) contains the full-length A β extracellular sequence, the APP transmembrane domain, and a few residues in the juxtamembrane sequence (KKK) of APP. These were cloned into a pET21a vector with an added N-terminal methionine and an attached linker/6 His-tag (KLAAALEHHHHHH) at the end of sequence for purification. We have previously shown that wild-type C55 and the C55* exhibit similar FTIR spectra arguing that the His tag is unstructured (8). In C99, the amino acids following the KKK sequence at the C-terminus are unstructured and extend into the cytosol (28). Consequently, in both C55 and C99, the hydrophilic C-terminus is not expected to influence the structure of the TM and extracellular domains.

The HSQC spectrum exhibits roughly one well-resolved resonance of equal intensity for each backbone and side chain NH group. The assignments were made on the basis of a series of three-dimensional NMR experiments and are in agreement with those determined for the full-length β -CTF (25,28) and a truncated β -CTF (29).

The chemical shifts of the glycine residues provide a potential probe of changes in C55 structure or dynamics. The GxxxG motifs mediate TM dimerization, whereas the G37-G38 sequence is thought to form a hinge facilitating substrate flexibility (31). Figure 3 presents the region of the 2D ^{15}N - ^1H HSQC spectra containing the glycine NH resonances of wild-type C55* and a series of Thr43 mutants in DPC micelles. The mutations

include T43A, T43V, T43I, T43M, T43F and T43G. The striking observation is that all the mutations influence the Gly37 and Gly38 resonances within the TM sequence. The T43V and T43I mutants, which involve a change to another β -branched amino acid, result in only changes to Gly37 and Gly38. The T43M and T43F mutants, which involve changes to large hydrophobic residues that are either flexible or relatively rigid, respectively, lead to additional shifts in Gly33. The T43G mutation is the most influential, leading to changes in all of the glycine residues except the N-terminal Gly9 resonance.

We previously observed differences between the structure of the extracellular domain of C55 in detergent and membrane bilayer environments (8). The hydrophobic L17-V18-F19-F20 sequence adopts helical secondary structure in detergent micelles, whereas it contributes to a small β -sheet domain that binds to the bilayer surface in a bilayer environment. The structure of the TM domain may be less sensitive to the change between detergent micelles and membrane bilayers than the extracellular sequence since the TM domain in both detergent micelles and bilayers resides within a hydrophobic environment. To probe if similar chemical shift changes occur at Gly37 and Gly38 in the T43I structure in bilayers, solid-state NMR measurements were undertaken by incorporating specific ^{13}C labels into C55 with solid-phase peptide synthesis. The C55 peptide was reconstituted into dimyristoylphosphocholine (DMPC), dimyristoyl-phosphoglycerol (DMPG) membrane bilayers to characterize the chemical shift changes between wild-type and T43I at residues Gly33, Gly37 and Gly38, as well as residues 39-42 and 44-45 that bracket position 43 in the sequence. The NMR chemical shifts of the ^{13}C labeled resonances were made using one-dimensional magic angle spinning (MAS) NMR. Table 1 summarizes the ^{13}C chemical shifts of the specific labeled amino acids within the TM region of C55. The glycine $^{13}\text{C}\alpha$ chemical shifts are in the range of 40-50 ppm, while $^{13}\text{C}=\text{O}$ chemical shifts are in the range of 174-180 ppm. Chemical shifts at the high ends of these ranges generally correlate with helical secondary structure, while chemical shifts at the lower ends of these ranges generally correlate with β -sheet secondary structure. The higher chemical shifts for both the

$^{13}\text{C}=\text{O}$ and the $^{13}\text{C}\alpha$ resonances in the TM glycine residues in the T43I mutant suggest that the structure of the TM domain is more helical than in wild-type C55. The increase in helical structure is illustrated by the plotting the chemical shift differences relative to random coil chemical shifts (Figure 4) where the more positive values in the T43I mutant relative to wild-type C55 reflect an increase in helical structure. There are larger changes in Gly37 and Gly38 compared to Gly33. Both ^{13}C resonances for Gly37 and Gly38 shift upfield by ~ 2 ppm. The large changes are Gly37 and Gly38 upon mutation agree with the solution NMR results showing that these glycines are the most sensitive to mutation.

The $^{13}\text{C}=\text{O}$ and $^{13}\text{C}\alpha$ chemical shifts of Val40, Ile41, Ala42, Val44 and Ile45 on the N-terminal side of Thr43 also exhibit changes characteristic of an increase in helical structure upon mutation (Figure 4). The only exceptions are the backbone $^{13}\text{C}=\text{O}$ of Val40 and the $^{13}\text{C}\alpha$ of Ile45. The Val40 $^{13}\text{C}=\text{O}$ has an unusually high chemical shift in wild-type C55 and is in a position to backbone H-bond to the Thr43 C β -OH. Loss of this interaction upon the T43I mutation would be expected to result in a lower frequency.

Together, the solution and solid-state NMR measurements suggest that global changes occur in the structure of C55 upon introduction of the T43I mutation. The chemical shift changes of the backbone ^{13}C resonances within the middle of the TM helix in the solid-state NMR experiments suggest that the helix is distorted in wild-type C55 and adopts a more canonical helical structure in the T43I mutant. We show below that the T43I mutation results in a shift in the monomer-dimer equilibrium toward monomer. Figure S1 (Supporting Information) presents overlays of the full HSQC spectra of the T43I and T43A mutants that illustrate large changes occur throughout the sequence, consistent with a dimer to monomer transition.

The T43I mutation alters the monomer-dimer equilibrium of C55—The dimer structure of the TM domain of APP has been reported in both detergent micelles (29,30) and in membrane bilayers (24). In DMPC:DMPG and POPC:POPS bilayers, the TM domain dimerizes via the $\text{G}^{29}\text{xxxG}^{33}\text{xxxG}^{37}$ interface (24). In contrast, the

G³⁸xxxA⁴² sequence was found to mediate dimerization in DPC detergent micelles (29). The GxxxA interface is the same as that predicted computationally in an earlier study by Gorman *et al.* (22) who proposed a comprehensive model for the influence of FAD mutations in cluster 2. In their studies using TM peptides, they showed that mutation of Thr43 shifts the structure of the TM dimer toward monomer. To test the dimer-to-monomer equilibrium in wild type C55 and in the T43I mutant, 2D solid-state NMR experiments were undertaken. The experiments were designed to establish if either the GxxxG or GxxxA dimer interface exists in the T43I mutant. These experiments involve co-mixing of two C55 peptides containing different ¹³C-labels and probing inter-helical magnetization transfer. Specifically, ¹³C labels were selectively incorporated at Gly33 and Ala42. Peptide 1 of the co-mixture contains ¹³C-carbonyl-labeled Gly33 and ¹³Cβ-labeled Ala42, while peptide 2 contains ¹³Cα-labeled Gly33 and ¹³C-carbonyl-labeled Ala42. In the C55 dimer, the Gly33 residues are expected to be closely packed if the G³³xxxG³⁷ motif mediates dimerization, whereas the Ala42 residues are in close proximity if the G³⁸xxxA⁴² motif mediates dimerization.

Figure 5 presents two-dimensional (2D) NMR measurements of potential GxxxG and GxxxA contacts using dipolar assisted rotational resonance (DARR), a solid-state NMR approach for determining through-space ¹³C...¹³C distances. Close proximity of ¹³C labels (< 6 Å) is manifested as a cross peak in the 2D NMR spectrum. Figure 5A shows the full 2D spectrum, where the region containing cross peaks is boxed in red. For the wild-type C55 peptide, the cross peak between the ¹³Cα carbon of Gly33 at 46.9 ppm and the ¹³C=O carbon at 174.2 ppm is observed (red box). A row through this region of the spectrum clearly reveals this cross peak (Figure 5B) and is consistent with a G³³xxxG³⁷ motif mediating dimerization. There is no cross peak between the Ala42 positions (8). In contrast, to the wild-type sequence, incorporation of the same ¹³C labeling scheme into peptides containing the T43I mutation does not result in interhelical cross peaks for either the GxxxG interface or the GxxxA interface (Figure 5C). We interpret the absence of cross peaks for either interface as a shift of the GxxxG dimer to

monomer, consistent with the previous FRET measurements of Gorman *et al.* (22).

Global Structural Changes in Helix Orientation and the Extracellular β-sheet—FTIR measurements of the frequency of the amide I vibration of TM peptides provides information on secondary structure, while measurement of the dichroic ratio provides information on the orientation of TM helices. Figure 6 presents FTIR spectra of wild-type C55* and a series of Thr43 mutations obtained by expression. The peptides were reconstituted into DMPC:DMPG membrane bilayers. The amide region of the spectrum exhibits an intense band at 1730 cm⁻¹ corresponding to the acyl chain carbonyls of the membrane lipids. The band at 1657 cm⁻¹ is characteristic of α-helical global secondary structure, while the band at 1626 cm⁻¹ band is characteristic of β-sheet secondary structure. The small 1695 cm⁻¹ band is often associated with anti-parallel β-sheet. We have recently assigned the β-structure in C55 to the extracellular sequence between Tyr10 and Ala21 (12). Upon mutation of Thr43, there is a general increase in resolution and an increase in intensity of the 1626 cm⁻¹ band. These changes indicate that mutations in the TM domain have an influence on the extracellular region, and suggest that the TM mutations induce a global change in structure.

Measurement of the dichroic ratio of the amide I normal mode using FTIR spectroscopy provides a probe of helix orientation relative to the membrane bilayer normal (Table 2). The amide I vibration is dominated by the stretching vibration of the backbone C=O group, which has an orientation roughly parallel to the helix axis. Polarized IR light is preferentially absorbed when the polarization is aligned parallel to the C=O bond. The dichroic ratio measures the intensity of absorbed IR light with orientations of 90° and 0° relative to the surface of the IR plate (see Methods). Polarized FTIR measurements were made on the TM sequence alone (residues 28-55) or on C55 (residues 1-55). The TM peptide has a high dichroic ratio of 3.5, consistent with an orientation that is nearly parallel to the bilayer normal. The TM domain of C55 exhibits a slightly smaller dichroic ratio (3.3). For the T43I, the dichroic ratios of the TM peptide and C55 mutants are less than the corresponding dichroic ratios of

the wild type peptides indicating a tilt away from the bilayer normal upon mutation.

As a result, the FTIR and NMR measurements argue that mutation of Thr43 in the middle of TM helix causes a change in the structure of C55 upstream of the site of mutation. A shift of the TM dimer to monomer would result in changes in the Gly33, Gly37 and possibly Gly38 chemical shifts and allow the helix to tilt more in the bilayer.

The T43I Mutation has a Stronger Influence on Processing than A21G in the A21G/T43I Double Mutant—We have recently characterized the structure and influence on proteolysis of the A21G Flemish mutation in the context of the C55 peptide (8). In comparison to wild-type C55 (or C99), the A21G mutation increases A β production by γ -secretase. This mutation reduces β -sheet structure of the extracellular sequence of C55 and increases α -helical structure from Gly25 to Gly29. The latter corresponds to a region near the membrane surface and thought to interact with cholesterol. In addition, the A21G mutation appears to slightly favor C55 dimerization (8). These effects are opposite to those seen for the T43I mutant.

In order to compare the relative influence of the A21G and T43I mutations, we examined processing of the β -CTF in the single mutants and together as a double mutant (Figure 7). We individually compared the production of A β 38, A β 40 and A β 42. The most significant changes are decreases of the amount of A β 40 and increases in the amount of A β 42 in both the single T43I mutant and the double A21G/T43I mutant. The relative concentrations of both A β 38 and A β 42 increase in the double mutant, A21G/T43I, relative to the single mutants.

The net result is that the T43I mutation has a stronger influence on processing than A21G in the A21G/T43I double mutant. Solid-state NMR of the double mutant of C55 indicate that the protein is monomeric (Figure 5E) suggesting that the conversion of dimer to monomer is driving the low production of A β 40 and high production of A β 38 in the double mutant. The individual mutations combine in an additive or synergistic

fashion to increase the amount of A β 42 in the double mutant.

Coupling of the T43I Mutation to Changes in the Extracellular Region of C55—The results above suggest that the influence of the T43I mutation on the extracellular region of C55 occurs via a shift toward a monomer. It has previously been shown that the extracellular sequence of C55 folds into β -sheet secondary structure and that this structure inhibits the production of soluble A β . The FTIR results in Figure 6 show that the β -sheet component of C55 increases in T43I and the other Thr43 mutants, consistent with the overall decrease in soluble A β . We have recently shown using fluorescence spectroscopy that the hydrophobic L17-V18-F19-F20 motif within extracellular β -sheet structure is inserted in bilayer and Phe19 penetrates into the head-group region of the bilayer (12).

In this section, we compare how the mutation of A21G and T43I influence membrane interactions using fluorescence spectroscopy of wild-type C55 and the A21G, T43I and T43I/A21G mutants. In each of these four peptides, Phe19 has been changed to tryptophan, whose fluorescence is highly sensitive to environment. Membrane embedded tryptophan generally exhibits a more intensive, blue-shifted fluorescence emission band than tryptophan in solution. This substitution does not change the β -sheet component of C55 in the context of the wild-type peptide. In the current studies, we find that the fluorescence intensity increases and there is a slight blue shift in the fluorescence maximum in the T43I and T43I/A21G mutants relative to wild-type C55 (Figure 8). In contrast, the fluorescence intensity decreases and there is a slight red shift in the fluorescence maximum the A21G C55. These changes are consistent with a more membrane buried tryptophan at position 19 in the context of the T43I mutant. We discuss below the potential effects of an increase in the β -sheet component in the T43I mutant.

DISCUSSION

Familial mutations in the APP gene fall into several clusters. One cluster is within the TM domain of the protein between Ala42 and Leu49, the γ - and ϵ -cleavage sites, respectively. This

cluster includes amino acids Thr43, Val44, Ile45, and Val46. We have targeted the T43I mutation and have shown that it induces global structural changes in the extracellular region of C55, as well as local changes in the region of Gly37 and Gly38. These changes are associated with a shift in the monomer – dimer equilibrium toward more monomer as first observed by Chakrabartty, Bowie and coworkers (22) and an increase in helical secondary structure near the γ -cleavage site. An analysis of the cleavage products resulting from γ -secretase processing of C99 shows that the T43I mutation reduces the total amount of secreted A β peptides mainly due to a decrease in A β 40, while increasing the A β 42/40 ratio. We hypothesize that the increase in helical secondary structure in monomeric A β peptide impedes enzyme-substrate interactions, substrate unraveling and proteolysis.

The T43I Mutation Causes Structural Changes Upstream of Thr43—Intramembrane cleavage of substrates by the γ -secretase complex is unusually slow (32,33). Moreover, it has not been established what distinguishes substrates from non-substrates other than the apparent requirement of extracellular domain shedding. For APP, the major γ -secretase cleavage sites within the β -CTF are at residues 38, 40 and 42. The TM cluster of FAD mutants occurs in the helical turn of amino acids that are just downstream of Ala42. De Jonghe *et al.* (34) found that T43I, V44M, V44A, I45V, V46I and V46L all increase the A β 42/40 ratio. Importantly, these mutations are all relatively conservative involving substitutions with different hydrophobic side chains. This observation suggests that non-conservative mutations prevent C99 processing. All mutations except I45V decreased A β 40 secretion, and all mutations except V44M increased the amount of secreted A β 42. These results suggested that there are local changes within the β -CTF substrate in the vicinity of the Val40 and Ala42 cleavage sites.

Moreover, there are several observations that imply that the FAD mutations within the TM domain can influence the structure of the γ -secretase substrate upstream of Thr43. For example, De Jonghe *et al.* (34) and Ancolio *et al.* (1999) found that mutations within the TM cluster of FAD mutants increased x-A β 42, where x-A β 42

are peptide isoforms that start with cleavage at amino acid Glu11 (alternative β -secretase cleavage) (1) or Leu17 (α -secretase cleavage) (15,35).

Our results provide support for structural changes both upstream (this section) and downstream (discussed in the following section) of Val40 or Ala42, the γ -cleavage sites. We further show that these changes are associated with a shift of the wild-type C55 dimer to monomer.

Both solution and solid-state NMR studies show that mutations at Thr43 influence the structure of the β -CTF in the region of the TM glycine residues (Gly33, Gly37 and Gly38), upstream of the γ -cleavage site. These glycines have been implicated in both dimerization of the TM domain and flexibility of the TM helix. For example, Munter *et al.* (36) found that mutations of Gly29 and Gly33 of the GxxxG motif gradually attenuate the strength of TM dimerization, reduce the formation of A β 42 and increase A β 38 and shorter A β species. The correlation between a reduction in dimerization and an increase in secreted A β 38 is similar to the results reported above.

One possibility is that dynamics of the monomer hinders the γ -secretase cleavage of C99 particularly to A β 40, and leads to a more equal distribution of A β species. Recent studies (37) indicate that the cluster 2 FAD mutations, including T43I, decrease the stability of the γ -secretase – substrate complex, consistent with a change in the structure or dynamics of the substrate.

The double glycine sequence (Gly37-Gly38) has also been implicated in forming a kink in the TM domain (31). Computationally, residues Val40 to Ala42 were found to be less α -helical than at the ϵ -cut site, and the side-chain hydroxyl of Thr43 was found to hydrogen bond to the backbone C=O of the i-4 residue in the TM domain (i.e. Val39) (31). Structurally, Bechinger and coworkers (23) found that the TM helix is disrupted around Ala42 and that this break provides flexibility for increased topological heterogeneity of the C-terminal domain. Their data is consistent with Gly37 mediating dimerization, with a kink or break in the TM helix occurring

below this point in the sequence. We find that Gly37 mediates TM helix dimerization in the wild-type peptide and that the region at and below Ala42 (i.e. Ala42, Val44, Ile45) becomes more helical in the T43I mutant, consistent with their observations.

Moreover, the disruption of the TM helix in the region of Ala42 may lead to hydrogen bonding of the Thr43 β -OH to the backbone C=O of Val40. This interaction may explain the unusual chemical shift of the $^{13}\text{C}=\text{O}$ group of Val40 and the strong influence of the Thr43 β -OH in modulating γ -secretase processing.

The FTIR data presented in Figure 2 also provide evidence for a change in the structure of the extracellular region of C55 upon mutation of Thr43. We observe reproducible changes in the intensity of the 1626 cm^{-1} band assigned to β -sheet structure between Tyr10 and Ala21. This structured region influences the level of A β 40 secretion by influencing γ -secretase cleavage (8). In particular, mutations of the L17-V18-F19-F20 sequence disrupt this extracellular β -sheet and are correlated with an increase in the secretion of A β peptides. Here, we observe the opposite effect, namely, the T43I, A, G and S mutants exhibit enhanced β -sheet in the extracellular domain of C55 and a reduction in A β 40 secretion. Monomer formation is associated with a change in the tilt of the TM helix away from the bilayer normal. This overall change in orientation of the substrate may reduce its ability to bind to the enzyme.

The T43I C55 Peptide is Monomeric—There has been considerable discussion about the whether C99 forms a monomer or dimer, and whether the oligomeric state influences γ -secretase processing (8,12,25,28,31,38-40). Our structural data indicating that the T43I mutation disrupts the wild-type C55 dimer agrees with fluorescence studies on TM peptides (22) and full length C99 (11) that the cluster 2 mutations all lead to a shift in the monomer-dimer equilibrium toward monomer.

This conclusion disagrees with solution NMR studies arguing that C99 is monomeric (28) or that TM peptides containing the V44M and V44A FAD mutations are dimeric (30). For C55, both the extracellular and TM domains influence

the overall structure of the β -CTF (8,12). Deletion or mutation of the extracellular domain as in the wild-type TM peptides shifts the monomer-dimer equilibrium toward dimer (8,12). For detergent solubilized C55, the level of detergent used can influence the monomer-dimer equilibrium, with less detergent driving peptide aggregation.

Importantly, the observation that monomer formation of the wild-type substrate decreases processing implies that the dimers are likely the native form of the substrate prior to complex formation. In fact, we previously found that the A21G mutation increases dimerization and increases the amount of secreted A β 40 (8,12).

Recent observations suggest that monomeric FAD mutant substrates having altered structures influence substrate binding and proteolysis even at the initial ϵ -cleavage step (37). The C99 monomer with an increase in helical secondary structure will likely behave more as a rigid rod and this may be more difficult to accommodate in the initial enzyme binding site or in the enzyme catalytic active site. The general change from dimer to monomer may then explain the decrease of total A β 40 production in T43I, as well as the other cluster 2 mutants.

Summary— γ -secretase catalysis of C99 involves at least four steps (1) substrate binding, (2) translocation to the active site, (3) catalysis, and (4) product release. Changes in substrate structure, dynamics and/or interactions at one or more of these steps may influence the amount and length of A β secreted by the enzyme.

First, the C99 substrate is in a monomer-dimer equilibrium in membrane bilayers where the structure, dynamics and membrane interactions of the substrate influence its initial binding to the enzyme complex away from the catalytic site (Figure 8B). As mentioned above, T43I C99 has been shown to have a weaker interaction with γ -secretase than wild-type C99 (37). The monomeric structure of the T43I mutant with a higher tilt angle of the TM helix and a more membrane-embedded extracellular domain may impede initial binding to the enzyme and be the origin of the lower levels of total A β peptide produced.

Second, the substrate is translocated to a position within the enzyme complex capable of

catalysis (41). Recent cryo-EM structures of the γ -secretase complex provide evidence for significant rearrangements of the TM helices during the catalytic process (42). The structure, flexibility and interactions of the substrate likely influence substrate translocation and the final orientation of the TM helix prior to the initial ϵ -cleavage.

Finally, progressive cleavage of the substrate is influenced by the ability of the substrate to unravel and form a productive catalytic complex with the enzyme (37). The wild-type dimer having distortions both in the region of Gly37-Gly38 and in the region below the γ -cleavage site likely facilitates unraveling. A rigid α -helix in the T43I monomer (as shown here) appears to decrease stability of the complex (37). Recent cryoEM structures of enzyme-substrate complexes with γ -secretase reveal a helical substrate that requires chemical cross linking to observe (43,44) possibly as a result of weak enzyme-substrate interactions.

EXPERIMENTAL PROCEDURES

Materials— ^{13}C -labeled amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA). Other amino acids and octyl- β -glucoside were obtained from Sigma Chemical (St. Louis, MO). DMPC and DMPG were obtained from Avanti Polar Lipids (Alabaster, AL) as lyophilized powders and used without further purification.

Peptide Synthesis and Purification—C55 peptides corresponding to the TM and JM regions of APP were synthesized by solid-phase methods (Keck Facility, Yale University). The purity was confirmed with MALDI mass spectrometry and analytical reverse phase HPLC.

Protein Overexpression and Purification—The 55 residues containing the full-length A β , transmembrane domain, and a few residues in the juxtamembrane sequence (KKK) of amyloid precursor protein are cloned into a pET21a vector with the added N-terminal methionine, and an attached linker/6 His-tag (KLAAALEHHHHHH) at the end of sequence. The plasmid harboring target DNA sequences then is transformed into BL21 *E. coli* strain, and plated

on Lauria-Bertani broth (LB)/ampicillin plates. A single colony on the plate is selected, and inoculated into 5 ml of LB/ampicillin medium containing overnight at 37 °C at 180 rpm (OD600 ~1.8). The cultured medium is spun down at 4 °C, and cells are re-suspended and cultured in 25 ml of sterilized LB medium or M9 minimal medium (40 mM Na_2HPO_4 , 20 mM KH_2PO_4 , 10 mM NaCl, 20 mM NH_4Cl , 0.2% glucose, and pH 7.0 supplemented with 0.1 mM CaCl_2 , 1 mM MgSO_4 , trace elements, and vitamin B) containing ampicillin (0.1 mg/ml), until the OD600 reaches 0.6. The cultured medium is inoculated to the sterilized 1 L of LB broth medium or (M9 minimal medium) at 37 °C at 180 rpm, until the O.D. reaches ~0.8. The cells are induced with 1 mM of isopropyl thiogalactoside (IPTG) for 16 h at 23 °C at 180 rpm. The induced cells (~1.5 gram) are then spun down at 6000 g, re-suspended in the 35 ml of ice-cold lysis buffer (75 mM Tris, 300 mM NaCl, 0.2 mM EDTA, and pH 7.8), and passed through a French pressure cell press (SLM AMINCO) twice at 1000 psi. The harvested cells are spun down at 25,000 g, and the pellets containing C55 are subjected to the subsequent purification.

The pellets are washed with the 35 ml of lysis buffer, and spun down at 25,000 g for 20 min till the washed solution becomes clear (usually with 3 iterations). The spun-down pellets are dissolved in 35 ml of urea/SDS buffer (20 mM Tris, 150 mM NaCl, 8 M urea, 0.2% SDS, pH 7.8). The pellet is first homogenized with an 18G 11/2 needle to increase the solvent exposed surface, and mixed well overnight at room temperature until the pellet is dissolved. The dissolved pellets are centrifuged at 25,000 g for another 20 min. The supernatants are pooled with the pre-equilibrium Nickel beads for 2 hours (2.5 mL of resin for 1 gram of cells pellet). The beads are then washed with 4 bed volumes of urea/SDS buffer, 4 bed volumes of SDS rinse buffer (20 mM Tris, 150 mM NaCl, 0.2% SDS, pH 7.8), and 8 bed volumes of detergent-contained Tris-buffered saline buffer (20 mM Tris HCl, 200 mM NaCl, pH 7.8) in a pulsed manner. The proteins are refolded, and eluted with 250 mM imidazole in Tris-buffered saline solution (pH 7.8) containing detergent. The eluted fractions are collected, and checked by SDS-PAGE. The protein concentration is estimated with 280 nm absorbance using an

extinction coefficient of $1490 \text{ M}^{-1} \text{ cm}^{-1}$, after removal of imidazole with an Amicon ultra spin concentrator (3 kDa molecular weight cut-off). The protein purity was further confirmed with mass spectroscopy (MALDI TOF).

Reconstitution of C55 into Membrane Bilayers—The C55 peptides were co-solubilized in DMPC, DMPG, and octyl- β -glucoside in hexafluoroisopropanol. The peptide: lipid molar ratio was 1:50; the molar ratio between DMPC:DMPG was 10:3. The solution was incubated for 90 min at 37°C , after which the solvent was removed under a stream of argon gas and then under vacuum overnight. 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (5 mM MES, 50 mM NaCl, pH 6.2) was added to the solid from the previous step and gently mixed at 37°C for 6 h. The octyl- β -glucoside was removed by dialysis (45). The reconstituted membranes were subject to FTIR analysis, and then pelleted and loaded into NMR rotors.

ATR FTIR Spectroscopy—Polarized attenuated total reflection (ATR) FTIR spectra were obtained on a Bruker IFS 66V/S spectrometer. ATR-FTIR spectroscopy is used to characterize the global secondary structure, and the orientation of TM domain of C55 in bilayers. The reconstituted peptides in bilayers are layer down on a germanium plate. The amide I vibrational frequency ($1600 - 1700 \text{ cm}^{-1}$) is sensitive to the secondary structure. An amide I frequency between $1650 - 1660 \text{ cm}^{-1}$ is characteristic of α -helix. The absorbance difference between 90° and 0° of polarized light provides information on the orientation of transmembrane helices relative to bilayers. The dichroic ratio (I_{90}/I_0) on TM helices is used to calculate the tilt angle, with the value of $\alpha = 41.8^\circ$ based on parallel measurements on bacteriorhodopsin (45).

Solution NMR Spectroscopy—NMR experiments were performed on a 700 MHz

Bruker AVANCE spectrometer. Several detergent systems (DPC, sodium dodecylsulfate, dihexoylphosphocholine, and octyl- β -glucoside) were screened to optimize the resolution in multi-dimensional experiments. TROSY (transverse-relaxation optimized spectroscopy) type of HNCO, CBCA(CO)NH and CBCANH spectrum were collected and analyzed for the sequential assignment. The sample temperature was maintained at 45°C for the full 3D experiments and 40°C for the HSQC spectra in Figure 3.

Solid-State NMR Spectroscopy—NMR experiments were performed at a ^{13}C frequency of 600 MHz on a Bruker AVANCE spectrometer. The MAS spinning rate was set to 9-11 KHz. The ramped amplitude cross polarization contact time was 2 ms. Two-pulse phase-modulated decoupling was used during the evolution and acquisition periods with the field strength of 80 kHz. Internuclear $^{13}\text{C}\dots^{13}\text{C}$ distance constraints were obtained from 2D DARR NMR experiments (46) using a mixing time of 600 ms. The sample temperature was maintained at 198 K. The solid-state MAS NMR spectra were externally referenced to the ^{13}C resonance of neat TMS at 0 ppm at room temperature. With TMS as the external reference, we calibrated the carbonyl resonance of solid glycine at 176.46 ppm. The chemical-shift difference between ^{13}C of DSS in D_2O relative to neat TMS was 2.01 ppm.

Processing of C99 and Thr43 Mutants—A β production from CHO cells was monitored in the cell culture media 48 h after transfection. Briefly, samples were cleared by centrifugation (12,000 g, 3 min, 4°C). A β 38, A β 40 and A β 42 were quantified in 25 μl of cellular medium by multiplex 4G8 or 6E10 A β ECLIA assays according to the manufacturer's instructions (MesoScale Discovery, Gaithersburg, MD).

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FOOTNOTES

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The abbreviations used are: A β peptide, amyloid β peptide; APP, amyloid precursor protein; ATR, attenuated total reflection; β -CTF, β C-terminal fragment; DARR, dipolar assisted rotational resonance; DMPC, dimyrstoyl-phosphatidylcholine; DMPG dimyrstoyl-phosphatidylglycerol; DPC, dodecylphosphocholine; FAD, familial Alzheimer's disease; FTIR, Fourier transform infrared; JM, juxtamembrane; MAS, magic angle spinning; TM, transmembrane

TABLE 1: Solid-State NMR Chemical Shifts within the TM Region of Wild-Type C55 and the T43I Mutant.

Residue	Peptide	$^{13}\text{C}=\text{O}$ (ppm)	$^{13}\text{C}\alpha$ (ppm)
Gly33	WT	174.2	46.9
	T43I	174.8	47.1
Gly37	WT	174.0	45.6
	T43I	176.8	47.7
Gly38	WT	174.2	45.8
	T43I	176.5	47.4
Val40	WT	179.8	62.6
	T43I	178.6	65.6
Ile41	WT	177.2	66.4
	T43I	177.5	66.8
Ala42	WT	177.4	55.6
	T43I	178.7	55.8
Val44	WT	178.6	66.9
	T43I	179.1	66.9
Ile45	WT	174.3	69.5
	T43I	176.8	66.6

TABLE 2: FTIR Dichroic Ratios of C55

Residue	Dichroic ratio-C55	Dichroic ratio-TM
Wild-type	3.3	3.5
A21G	3.3	3.4
T43I	2.9	3.1
A21G/T43I	3.0	N.D.
LVFF/AAAA	3.5	N.D.

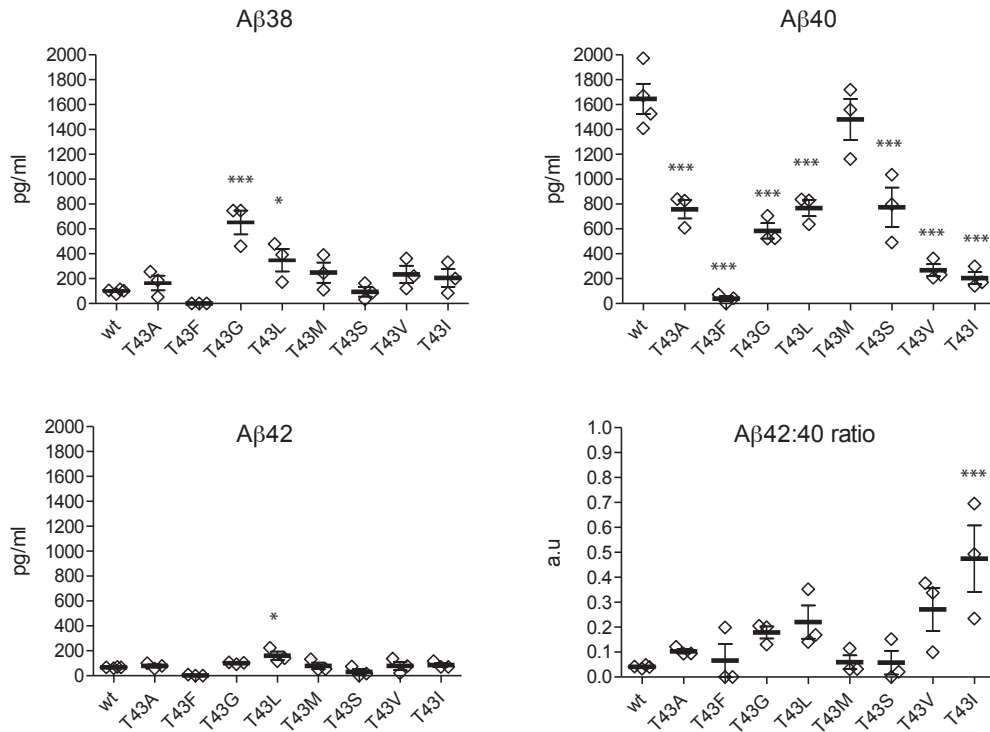
Figure 1

FIGURE 1. Differences in Aβ production upon mutation of Thr43. DNA constructs of wild-type C99 and the T43 mutants were transfected into Chinese hamster ovary (CHO) cell cultures. These constructs express at comparable levels. The secretion of Aβ38, Aβ40 and Aβ42 were measured by multiplex 4G8 or 6E10 Aβ ECLIA assays (MesoScale Discovery, Gaithersburg, MD). The Aβ42/40 ratio increases 10.5 and 7.2-fold in T43I and T43V mutants, respectively, relative to the wild-type peptide. The Aβ42/40 ratio also increases 4.3 and 5.2-fold in the T43G and T43L mutants, respectively, compared to wild-type. Overall, the mutations on Thr43 increase Aβ42/40 ratio by decreasing the Aβ40 production. The statistics are based on a one way analysis of variance using a Dunnett post hoc test (all columns are compared to wild-type). Number of samples: N=4 for wild-type; N=3 for mutants. The different samples (N) correspond to biological replicates in different cultures. In each culture, we pooled n=3 (different wells) for each condition (wt, mutants) * p<0.05, ** p<0.01, and *** p<0.001.

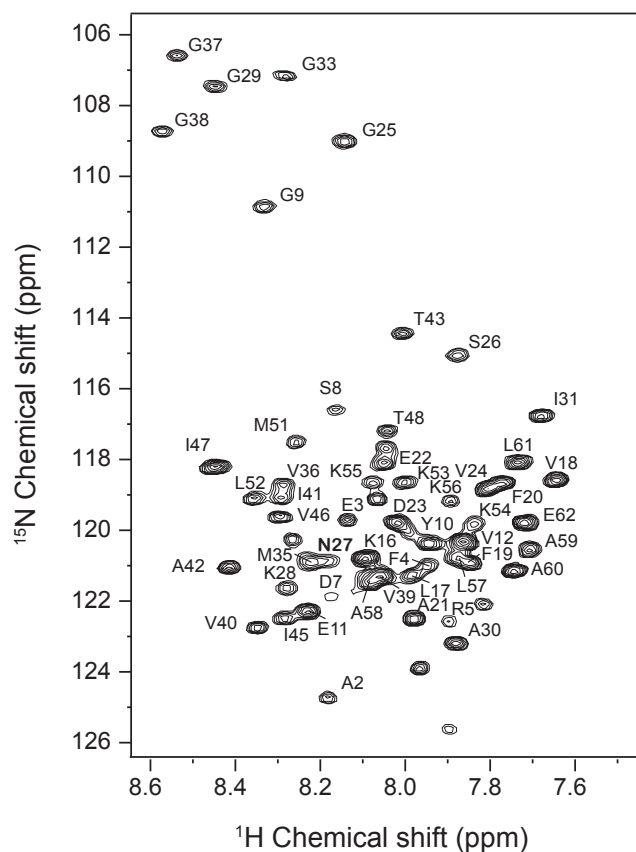
Figure 2

FIGURE 2. ^1H - ^{15}N -HSQC spectrum of wild-type C55* solubilized into DPC micelles. Sequential backbone assignments were made on the basis of a series of TROSY-type three-dimensional NMR experiments. The 2D NMR data were collected at 45 °C.

Figure 3

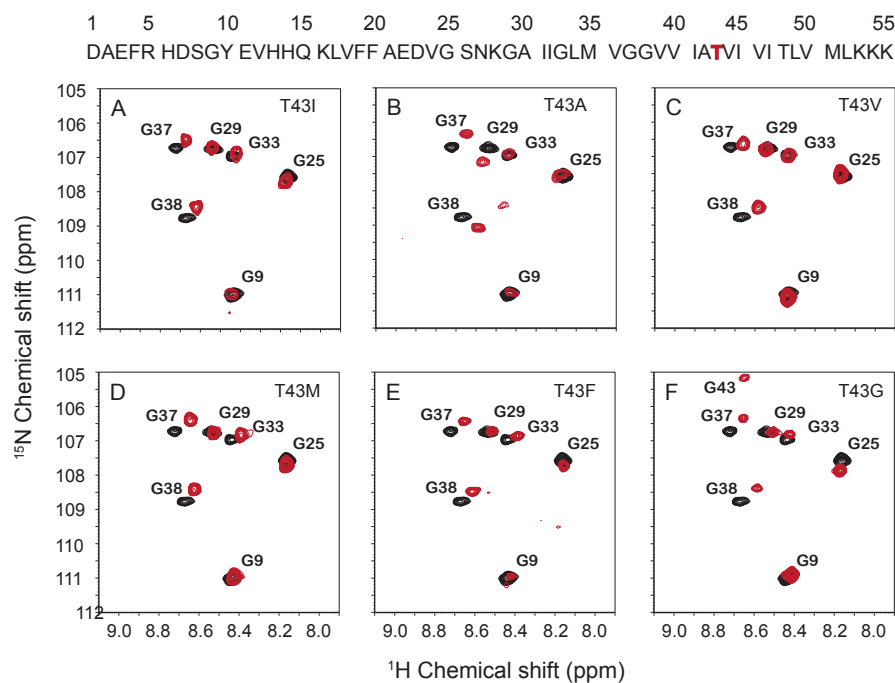


FIGURE 3. Comparison of ^1H - ^{15}N -HSQC spectra of wild-type C55* (black) and selected Thr43 mutants (red). The Thr43 mutations (T43I, T43A, T43V, T43M, T43F, and T43G) cover a range of different side chain types. Only the glycine region of the HSQC spectrum is shown to highlight the strong influence of the Thr43 mutations on the Gly37 and Gly38 chemical shifts. Data were obtained at 700 MHz at a temperature of 40 °C. The sequence of C55 is shown at the top of the figure.

Figure 4

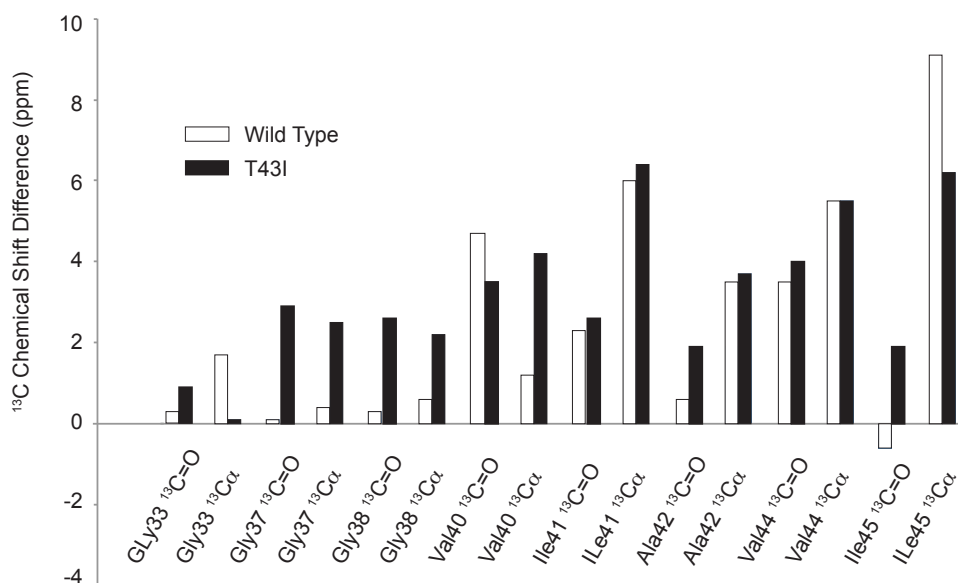


FIGURE 4. Chemical shift differences for the backbone $^{13}\text{C}=\text{O}$ and $^{13}\text{C}\alpha$ NMR resonances in wild-type (white) and T43I (black) relative to random coil values (http://www.bmrb.wisc.edu/published/Ikura_cs_study/part2.html). A positive change is associated with an increase in helical secondary structure.

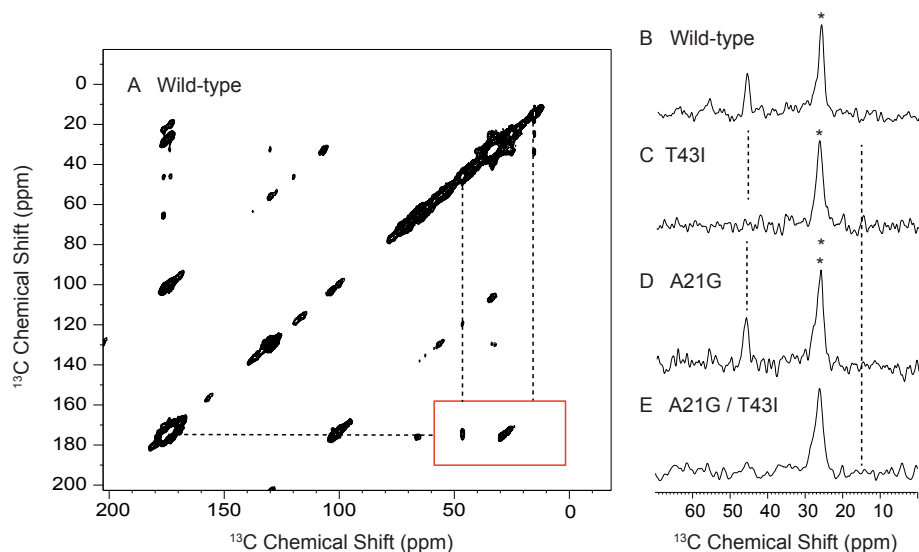
Figure 5

FIGURE 5. Dimer contacts between TM helices of C55 probed using 2D DARR NMR spectroscopy. DARR NMR spectra were obtained of C55 peptides corresponding to the WT sequence (*A,B*) T43I (*C*), A21G (*D*), and A21G/T43I (*E*). Panel *A* shows the full 2D spectrum. Panels *B-E* show the rows through the boxed region in panel *A*. For these measurements, ^{13}C -carbonyl and ^{13}C -C α labels were separately incorporated at Gly33 in two C55 peptides. The peptides were co-mixed in equimolar ratios and reconstituted into membrane bilayers (*A-E*). Cross peaks are present as a result of interhelical dipolar couplings. In order to rule out the possibility that the cross peaks observed originate from ^{13}C labels within a single peptide (i.e. intrahelical dipolar couplings between the Gly33 C α and Ala42 C=O), we also reconstitute the peptides individually into membrane bilayers and run parallel experiments. Neither peptide that we use in our co-mixture exhibits intramolecular cross peaks when measured separately.

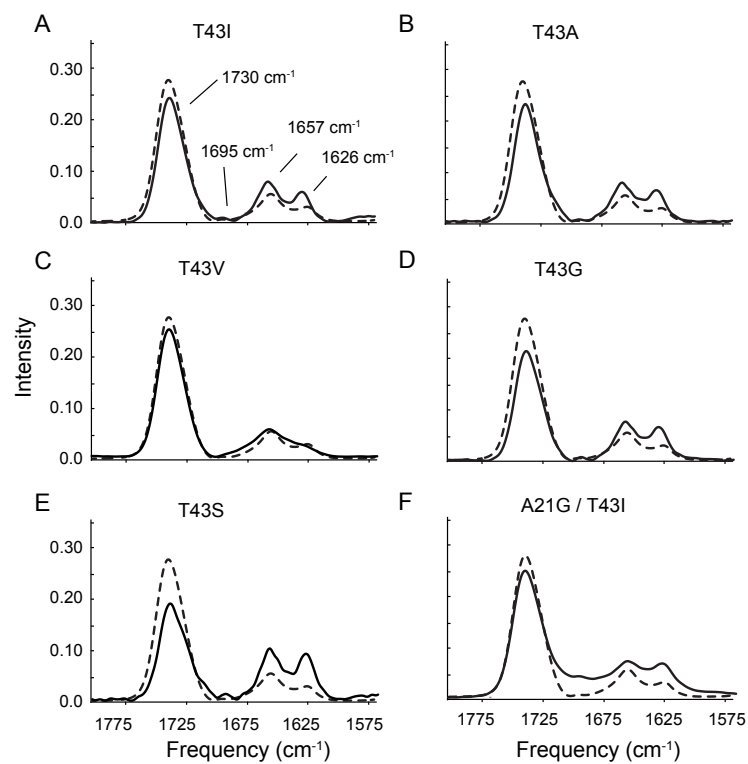
Figure 6

FIGURE 6. FTIR spectra of the amide I region of wild-type C55* (dashed line) and several Thr43 mutants (solid lines). The peptides were reconstituted into DMPC:DMPG membrane bilayers in a 1:50 peptide-to-lipid molar ratio.

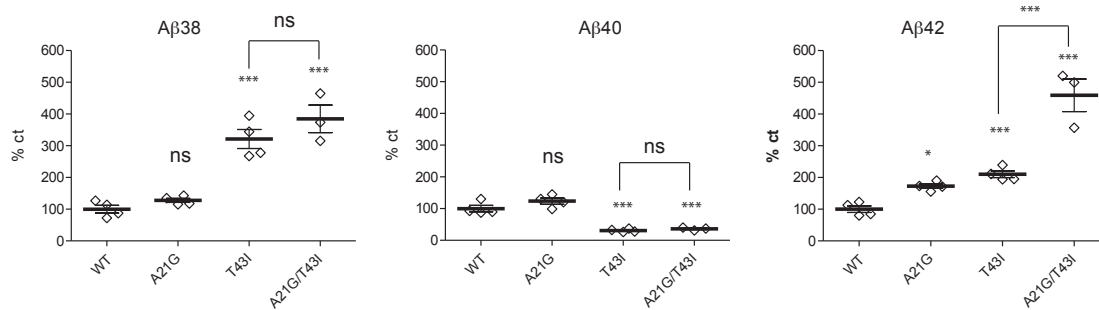
Figure 7

FIGURE 7. Production of Aβ peptides resulting from γ-secretase cleavage of wild-type C99 and C99 containing the A21G, T43I and A21G/T43I mutations. The percentage Aβ38, Aβ40 and Aβ42 were all normalized to the relative production from wild-type C99. The statistics are based on a one way analysis of variance using a Dunnett post hoc test (all columns are compared to wild-type). Number of samples: N=4; except for A21G/T43I (N=3). The different samples (N) correspond to biological replicates indifferent cultures. In each culture, we pooled n=3 (different wells) for each condition (wt, mutants). * p<0.05, ** p<0.01, and *** p< 0.001. ns – not significant.

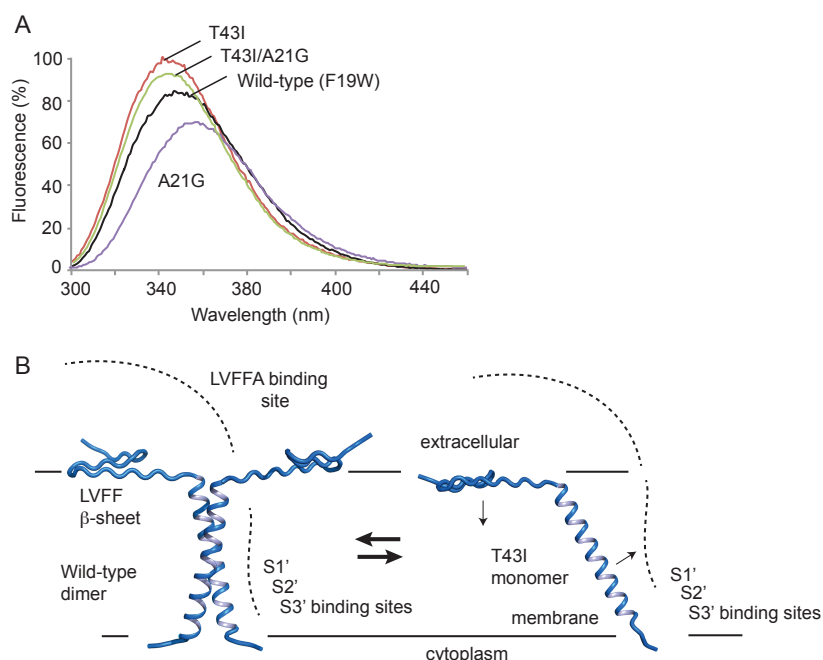
Figure 8

FIGURE 8. *A*, Fluorescence spectroscopy of the F19W mutation reveals changes in membrane association of the extracellular β -sheet domain of C55. Tryptophan fluorescence spectra are shown of wild-type C55, along with C55 containing the A21G, T43I and A21G/T43I mutations. The wild-type C55, T43I C55 and A21G/T43I C55 have shifted fluorescence spectra. The spectrum of A21G is more similar to that of the F20W mutant (12). These spectra were obtained with a 200:1 lipid to protein molar ratio. Experiments with 300:1 and 100:1 gave similar results. *B*, Schematic showing the proposed structural changes that result from the A21G and T43I mutations. The wild-type C55 peptide forms a dimer mediated by its TM domain with the extracellular region adopting β -sheet structure that lies on the membrane surface. The T43I mutant leads disrupts the TM dimer. The TM helix tilts $\sim 10^\circ$ in the membrane and the extracellular β -sheet becomes more membrane embedded. Both the extracellular and TM regions have binding sites in the γ -secretase complex (dashed line). A recent cryo-EM structure (50) of the substrate in complex with γ -secretase exhibits an unraveled C-terminus and extended extracellular domain in agreement with previous NMR and FTIR studies of membrane embedded C55 (8,12).

Influence of the familial Alzheimer's disease-associated T43I mutation on the transmembrane structure and γ -secretase processing of the C99 peptide

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