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Impact of Amyloid Precursor Protein Hydrophilic Transmembrane Residues on Amyloid-Beta Generation

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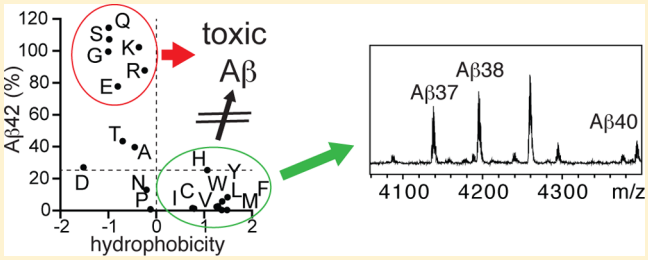
ABSTRACT: Amyloid-β (Aβ) peptides are likely the molecular cause of neurodegeneration observed in Alzheimer's disease. In the brain, Aβ42 and Aβ40 are toxic and the most important proteolytic fragments generated through sequential processing of the amyloid precursor protein (APP) by β- and γ-secretases. Impeding the generation of Aβ42 and Aβ40 is thus considered as a promising strategy to prevent Alzheimer's disease. We therefore wanted to determine key parameters of the APP transmembrane sequence enabling production of these Aβ species. Here we show that the hydrophilicity of amino acid residues G33, T43, and T48 critically determines the generation of Aβ42 and Aβ40 peptides (amino acid numbering according to Aβ nomenclature starting with aspartic acid 1). First, we performed a comprehensive mutational analysis of glycine residue G33 positioned within the N-terminal half of the APP transmembrane sequence by exchanging it against the 19 other amino acids. We found that hydrophilicity of the residue at position 33 positively correlated with Aβ42 and Aβ40 generation. Second, we analyzed two threonine residues at positions T43 and T48 in the C-terminal half of the APP-transmembrane sequence. Replacement of single threonine residues by hydrophobic valines inversely affected Aβ42 and Aβ40 generation. We observed that threonine mutants affected the initial γ-secretase cut, which is associated with levels of Aβ42 or Aβ40. Overall, hydrophilic residues of the APP transmembrane sequence decide on the exact initial γ-cut and the amounts of Aβ42 and Aβ40.



Alzheimer's disease (AD) is a chronic illness that evolves over decades before clinical symptoms occur.[1,2] First molecular changes during the presymptomatic phase are elevated amyloid-beta (Aβ) levels in sporadic and inherited forms of AD as early as 15−20 years before symptomatic onset of AD.[1−4] Aβ peptides readily assemble into soluble oligomers, which are most toxic to neurons as compared to monomers or fibrils, and likely initiate the neurodegeneration underlying AD.[5−8] The length of Aβ varies between approximately 31 and 49 amino acids; however, peptides with 42 amino acids (Aβ42) are more toxic than shorter forms with 40 or 38 (nontoxic) residues.[9] Aβ peptides derive from proteolytic processing of a larger amyloid precursor protein (APP).[10] During the amyloidogenic processing, the ectodomain of APP is shed off by β-secretase, a membrane-bound aspartic-acid protease. The membrane-residing C-terminal fragment (CTF) of APP becomes then a substrate for γ-secretase, an intramembrane protease with at least four subunits cleaving within the APP transmembrane sequence (TMS).[11] The diversity in lengths of Aβ peptides results from sequential proteolytic cleavages by the γ-secretase complex starting at the cytoplasmic end of the APP TMS and progressing stepwise by three to four amino acids toward the N-terminal end of the TMS.[12−14] This mechanism is also described by the term γ-secretase processivity.[15] The APP TMS is processed in two predominant degradation pathways, also termed product lines. These are the Aβ40 product line encompassing Aβ49−Aβ46−Aβ43−Aβ40−Aβ37 and the Aβ42 product line with Aβ48−Aβ45−Aβ42−Aβ38.[12−14] The first cut occurs either at position 49 or 48, and commits APP to one of the product routes. Although switches between the two product lines are possible, they are rare.[12] The importance of detailed knowledge about APP processing is reinforced by recent genetic analyses, which identified further AD risk loci associated with APP pathways.[16] Furthermore, subjects carrying an APP mutation leading to reduced Aβ generation are protected from AD.[17] Thus, lowering Aβ generation is regarded to ameliorate or prevent AD pathology.[18] Understanding the detailed molecular mechanisms of Aβ generation would therefore enable rational approaches and stimulate new strategies to intervene with production of toxic Aβ species. We herein investigated the effect of polar residues within the APP TMS on the proportion of Aβ42 and Aβ40 generation.

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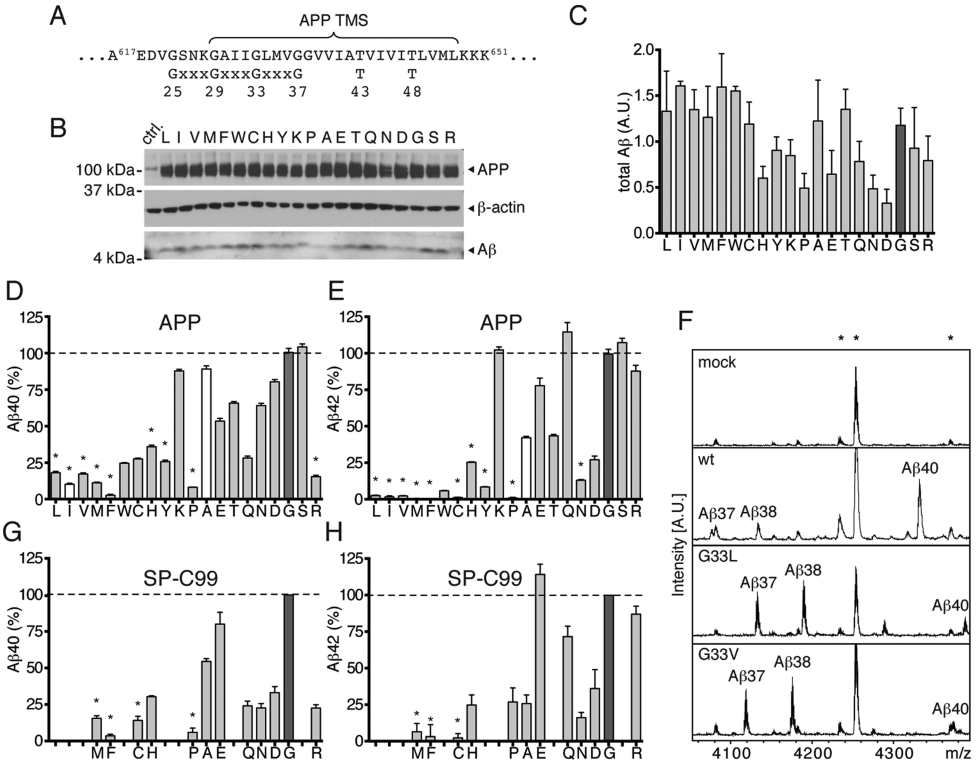

Figure 1. Aβ generation of G33 mutants. (A) APP sequence with highlighted hydrophilic residues glycine G25, G29, G33, G37 as part of the GxxxG motifs and residues T43 and T48. The TMS spans amino acids glycine 625 - leucin 648 (29−52 in Aβ nomenclature). (B) Western-blot analysis of APP-G33 mutants transiently expressed in SH-SY5Y cells. APP and β-actin were detected from cell lysates using 22C11 and C4 antibodies, respectively. Aβ was detected directly from cell culture supernatant using W0-2 antibody. Representative images of three independent experiments. (C) Densitometric quantification of total Aβ levels from Western blots using the antibody W0-2. In each experiment, the strongest Aβ signal was set to 1 in comparison to the other Aβ signals, and three independent experiments were averaged. No significant differences were observed using the Kruskal−Wallis test and Dunn's multiple comparisons test (n = 3). A.U.: arbitrary units. (D, E) Aβ40 and Aβ42 levels of APP-G33 mutants were quantified by sandwich ELISA (mean ± SEM, n = 3−7), wild type (G33) normalized to 100%, Dunn's multiple comparisons test: $p^* < 0.05$. Note that mutants G33A and G33I were previously published,[26] but reanalyzed herein (white bars). (F) MALDI-MS spectra of Aβ peptides generated from APP wt, G33L, and G33V. Note that the Aβ-mass shifts are due to the amino-acid substitutions. Shown are representative spectra from three independent experiments. A.U.: arbitrary units; Ctrl.: control; m/z: mass per charge. Stars (*) label peaks not related to Aβ. (G, H) Aβ40 and Aβ42 levels of SP-C99-G33 mutants were quantified by sandwich ELISA (mean ± SEM, n = 3−7), wild type (G33) normalized to 100%, Dunn's multiple comparisons test: $p^* < 0.05$.

TMSs are mainly composed of hydrophobic amino acids, which facilitate integration into the hydrophobic environment of the membrane. The four most common residues are accordingly leucine, isoleucine, valine, and alanine, while residues with medium polar or polar side chains are less frequent in TMSs.[19] They often contribute a particular function or promote protein−protein interactions. As a prominent example, signaling of the erythropoietin receptor is modified through a serine residue (S238) that associates its TMS with the TMS of the viral protein Gp55.[20] Also, the mutation of V466 to the hydrophilic glutamic acid within the TMS of the erbB2 receptor enhances its TMS interactions and causes the receptor to be constitutively activated.[21] Generally, hereditary human diseases are often caused by mutations affecting the polarity/hydrophobicity of TMS residues, presumably by changing the TMS stability or oligomerization status.[22] On the basis of the importance of hydrophilic residues in TMSs, we hypothesized that the hydrophilic residues of the APP TMS determine Aβ generation. We focused on the following hydrophilic positions: First, we analyzed one glycine residue G33 within the N-terminal APP-TMS region. The absence of a side chain at Gly33 leaves the polar main chain atoms unshielded from the environment resulting in a local polarity; thus glycine is considered as a polar amino acid.[23] Second, we investigated two threonine residues T43 and T48 in the C-terminal APP-TMS region, where familial AD mutations are found clustered.[24] We exchanged G33 with all other 19 standard amino acids and found that the hydrophobicity of the amino acid replacement is a key parameter determining Aβ generation in position 33. Exchange of threonine residues revealed that they decide on the Aβ product line in which the APP TMS is degraded. Taken together, our results show that hydrophilic amino acids G33, T43, and T48 of the APP TMS determine the exact initial γ-cut and the amounts of Aβ42 and Aβ40 produced.

EXPERIMENTAL PROCEDURES

Cell Culture. SH-SY5Y cells (ATCC number: CRL-2266) were maintained in DMEM/Ham's F12 medium containing 10% FCS, 20 mM glutamine, 20 mM sodium pyruvate, 1× nonessential amino acids. For transient or stable expression, plasmids (2 μg) were transfected into SH-SY5Y cells using the transfection reagent TransFectin (Bio-Rad) following the manufacturer's instructions. For stable expression of constructs, the medium was complemented with 270 μg/mL hygromycin.

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Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) and Western blotting. Stably or transiently transfected SH-SY5Y cells were plated at a density of 3.6 × 10^5 cells per 12-well dish. The day after splitting, 600 μL of fresh medium was incubated for 24 h. For Aβ40- and Aβ42-specific ELISAs, 50 μL of medium was analyzed using G2-10 and G2-13 as capture antibodies and W0-2-biotin as detection antibody. For Western blot analysis, Aβ and sAPPα were detected from conditioned medium with the antibody W0-2 and sAPPβ with a specific antibody JP18957 (IBL). For detection of APP, SP-C99 and actin by Western blot, cells were lyzed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, and complete protease inhibitor containing EDTA (Roche). Antibodies directed against APP (22C11), β-actin (C4, Millipore), and APP CTF (171610, Millipore) were used.

γ-Secretase in Vitro Assay. Confluent SH-SY5Y cells stably expressing APP695 wt or mutants (N-terminal Myc and C-terminal Flag tag) were homogenized in buffer A (20 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 140 mM KCl, 250 mM sucrose, 5 mM EGTA, 1× complete protease inhibitor containing EDTA (Roche), pH 7.0). Cell homogenates were centrifuged at 500*g*. The supernatant was further centrifuged at 100000*g* for 1 h to obtain the total membrane fraction. The membrane pellet was suspended in buffer B containing 50 mM PIPES, 250 mM sucrose, 1 mM EGTA, 1× complete protease inhibitor with EDTA, pH 7.0; protein concentration adjusted to 10 mg/mL. An equal volume of buffer B with 2% 3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO) was added and incubated for 1 h on ice to solubilize the membranes. Insolubilized membranes were removed by centrifugation at 100000*g* for 1 h. Solubilized membranes were diluted 1:4 with buffer B, 10 mg of total protein per reaction, and incubated for 3 h at 37 °C. CTFs, APP and Aβ were removed by immunoprecipitation with 30 μL of protein-G-sepharose and 5 μg of 4G8 antibody for 2 h. AICD was precipitated from the supernatant in a second immunoprecipitation, using 30 μL of protein-G sepharose and 5 μg of anti-Flag M2 antibody (Sigma) overnight at 4 °C. Beads were washed twice with PBS and 100 mM ammonium acetate; proteins were eluted with 50% acetic acid and dried by vacuum centrifugation.

Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) for Aβ and AICD Detection. Aβ peptides were immunoprecipitated from 24 h conditioned cell-culture supernatant in a similar immunoprecipitation procedure as for AICD outlined above, but with 30 μL of protein-G sepharose and 5 μg of W0-2 overnight. Dried Aβ or AICD peptides were solubilized in 10 μL of 33.3% acetonitrile, 0.1% TFA by sonication for 15 min. One microliter sample was spotted with 1 μL of sinapinic acid using the dried-droplet method and measured using a MALDI-MS Bruker Ultraflex II TOF/TOF or UltrafleXtreme (Aβ of threonine mutants) in reflector or linear mode.

Plasmids and Generation of Mutants. APP695 (N-terminal Myc and C-terminal Flag tag) or the C-terminal fragment of APP (SP-C99) with a signal peptide (SP), SP-C99 (C-terminal Flag tag) were used as a template to introduce Gly33 mutations by site-directed mutagenesis. The cDNAs were inserted into pCEP4 (Invitrogen), for full-length APP or pcDNA3.1 (Invitrogen) for SP-C99. SP-C99 contained the Asp-Ala linker amino acids as previously published.[25] All sequences were confirmed by dideoxy sequencing.

Software and Statistical Analysis. Statistical analyses were performed using Graphpad Prism 6. Because of the non-normal distribution of our data, we used the Kruskal–Wallis test with Dunn's multiple comparisons. Figures were prepared using GraphPad Prism 6, Adobe Photoshop CS6, and Adobe Illustrator CS6. Densitometric quantification of Western blots was performed using ImageJ 1.47v.

RESULTS

Processing of APP G33 Mutants. Four glycine residues G29, G33, G37, and G38 are found in the N-terminal region of the APP TMS (Figure 1A). G33 is in a central position in the middle of this glycine zipper motif and was previously shown to affect Aβ production.[26,27] We replaced G33 against all other 19 standard amino acids and expressed the APP mutants in SH-SY5Y cells to analyze the impact of G33 mutations on Aβ production.

We found a trend toward reduced total Aβ levels for the mutants G33H, P, D, and N as determined by Western blot analysis. All other mutants produced total Aβ levels comparable to wild type APP (Figure 1B,C). To quantify the two most relevant Aβ species Aβ40 and Aβ42, we applied ELISA (Figure 1D,E). In these analyses, G33D and N showed strong signals for at least one of the two species Aβ40 and Aβ42. In general, G33 mutants caused Aβ40 levels to vary between 3% and 104% compared to wild type, with the phenylalanine substitution having the strongest effect and preventing Aβ40 production almost completely (Figure 1D). More than half of the mutants generated less than 30% Aβ40 compared to wild type. Similarly, half of the G33 mutants produced less than 10% Aβ42 (Figure 1E). Only five mutants G33R, Q, E, K, and S showed more than 75% Aβ42 compared to the wild type indicating that Aβ42 levels are more sensitive to G33 substitutions than Aβ40 levels. Comparison of the effects of G33 point mutations on Aβ42 and Aβ40 levels reveals two striking features: First, G33 mutants that decreased Aβ40 levels, generally also decreased Aβ42 levels, except for G33Q and G33R. The latter mutant dramatically decreased Aβ40 levels, but Aβ42 levels remained at comparable levels to the wild type. This unusual Aβ pattern reflects an overall shift in processing away from the Aβ40 product line and was further analyzed as outlined below. Second, the substitutions L, I, V, M, and F at G33 generated negligible amounts of Aβ40 and Aβ42 as quantified by ELISA; however total Aβ levels remained at the wild type level as determined by Western blot (compare Figure 1C with Figure 1D,E). In agreement with previous studies, these mutants elevate levels of shorter Aβ species Aβ38 and Aβ37 as shown for G33L and G33V by mass spectrometry (Figure 1F).[26,28,29] Of note, the observed mass shifts of 56 and 42 Da match the values expected for a glycine-to-leucine and glycine-to-valine substitution, respectively.

Next, to exclude effects of APP-G33 mutations on β-secretase cleavage efficiency, we selected 11 G33 mutations, particularly those which showed lower Aβ levels, and introduced them into SP-C99, a construct corresponding to the C-terminal fragment of APP (SP-C99) after β-secretase cleavage.[25] Aβ40 and Aβ42 levels generated from SP-C99 mutants were grossly similar to Aβ levels generated from APP-G33 mutants (Figure 1G,H), indicating that G33 replacements affect principally γ-secretase, but not β-secretase cleavage.

Aβ Generation Is Linked to Amino-Acid Hydrophobicity at Position 33. To rationalize the observed effects of each of the G33 mutations on Aβ40 and Aβ42, we related

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the Aβ40 and Aβ42 levels to biophysical properties of the amino acids used for substitution applying various propensity scales. A systematic pattern was observed for amino-acid hydrophobicity. Since the APP TMS has an α-helical structure, we preferred to use the $\alpha\alpha$-hydrophobicity scale published by Cid et al.[30] This scale is based on the original scale by Ponnuswamy et al.,[31] but here the hydrophobicity values were calculated only from α-helical proteins. The hydrophobic trait of most amino acids remains unchanged in this scale; however, for alanine the steric factors in α-helices overcome the chemical features rendering alanine a hydrophilic amino acid.[30] Hydrophobic amino acids in position G33 drastically diminish Aβ40 and Aβ42 generation, whereas hydrophilic amino acids promote Aβ generation (Figure 2A,B). Likewise, the Aβ levels

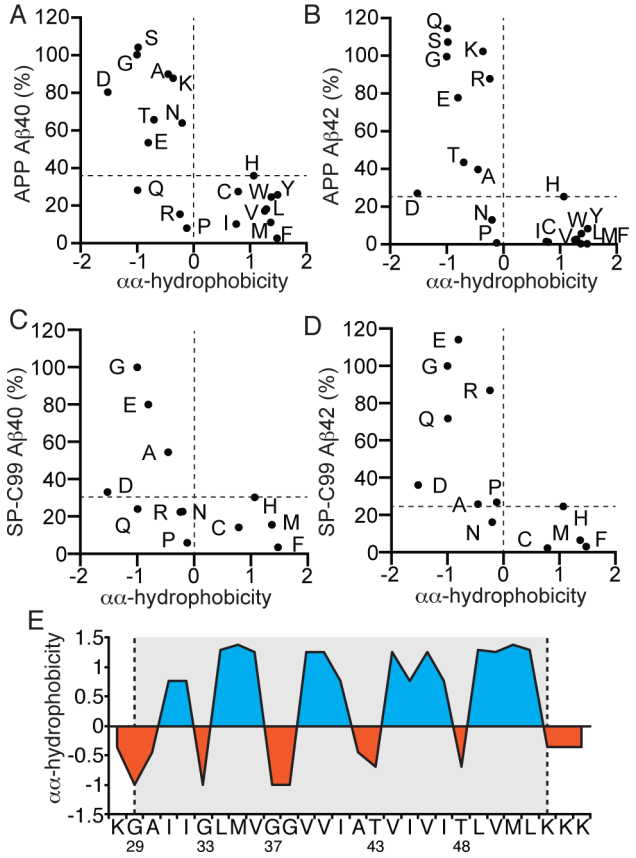

Figure 2. Amino-acid hydrophobicity affects Aβ generation. Aβ40 (A) and Aβ42 (B) levels generated from APP mutants as well as Aβ40 (C) and Aβ42 (D) levels generated from SP-C99 mutants were correlated with the $\alpha\alpha$-hydrophobicity values according to Cid et al.[30] Horizontal and vertical dashed lines highlight formation of mutation subgroups. (E) Hydrophobicity plot of the wild type APP TMS according to the Cid scale highlighting hydrophilic residues. The residues are numbered according to Aβ nomenclature.

generated from SP-C99 G33 mutants basically revealed the same correlation with hydrophobicity (Figure 2C,D). We observed two groups of G33 mutants: Group A comprising hydrophilic amino acids enabling Aβ generation (A, D, E, K, S, T as well as Q and R for Aβ42) and group B comprising hydrophobic amino acids preventing Aβ generation (C, F, H, I, L, M, V, W, Y). Thus, hydrophobicity (or vice versa polarity) at position G33 strongly determines the generation of Aβ40 and Aβ42. To analyze whether the hydrophobicity of amino acids at other positions of the APP TMS has a similar effect on Aβ production, we next investigated substitutions of T43 and T48 in the C-terminal region of the APP TMS (Figure 2E).

Impact of APP TMS Threonine Residues on Aβ Generation. To investigate the effects of the hydrophilic side chains at T43 and T48 on the production of Aβ40 and Aβ42, threonines were exchanged against valines (T43V, T48V, and T43/48V), one of the most hydrophobic amino acids. Aβ40 generation was almost completely suppressed by T43V, but Aβ42 levels were increased to 160% of the wild type, similarly to the familial AD mutation T43I[27,32] (Figure 3A). By

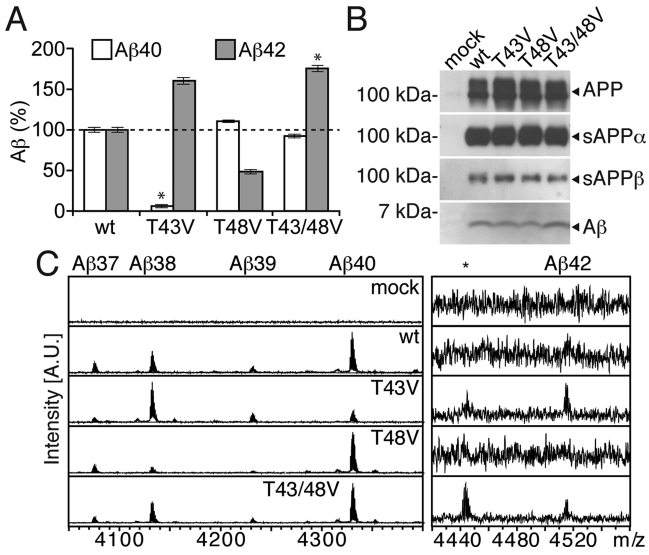

Figure 3. Processing analysis of APP-threonine mutants. (A) Aβ40 and Aβ42 quantification by ELISA. All values are represented as mean ± SEM, n = 5−7, Dunn's multiple comparisons test: $p^* < 0.005$. (B) APP-expression control from cell lysates (22C11) and secreted fragments from cell-culture supernatant by Western blot (sAPPα and Aβ: W0-2; sAPPβ: IBL). Representative images of three independent experiments. (C) MALDI-MS analysis of secreted Aβ species; A.U.: arbitrary units; m/z: mass per charge. Shown are representative spectra from three independent experiments. Stars (*) label peaks not related to Aβ.

contrast, T48V located at the opposite side of the APP-TMS helix caused inverse effects, though not as pronounced: Aβ40 levels remained unchanged, but Aβ42 levels dropped to 49% compared to the wild type. We expected the double mutant T43/48V to resemble either one of both single mutants, with one of the threonines dominating APP processing. What we observed, however, was that Aβ40 levels of the T43/48V double mutation did not change, but Aβ42 increased to 175% of the wild type, thus approximately reproducing Aβ40 levels of the T48V mutant and Aβ42 levels of the T43V mutant (Figure 3A). These mutations did not affect overall APP processing (Figure 3B). MALDI-MS analysis of secreted Aβ peptides showed that the decrease of Aβ40 by T43V coincides with increased Aβ42, Aβ39 and Aβ38 levels, strongly indicating that γ-secretase processing favored the Aβ42 product line over the Aβ40 product line (Figure 3C). Similarly, the decrease of Aβ42 by T48V also causes a decrease in Aβ38 levels, indicating that overall less processing occurred via the Aβ42 product line. The results imply that threonine mutations restrict processing to either Aβ product line. Or vice versa, if only one threonine residue is present, only one Aβ product line is favored.

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APP Mutants with Impact on the ε-Cut. The first cut by γ-secretase was initially named ε-cut[33] and occurs between amino acids 48−49 or 49−50 resulting in two APP intracellular domains (AICD), i.e., the AICD49 or AICD50, respectively (Figure 4A). The cut between 48 and 49 results in Aβ42 and

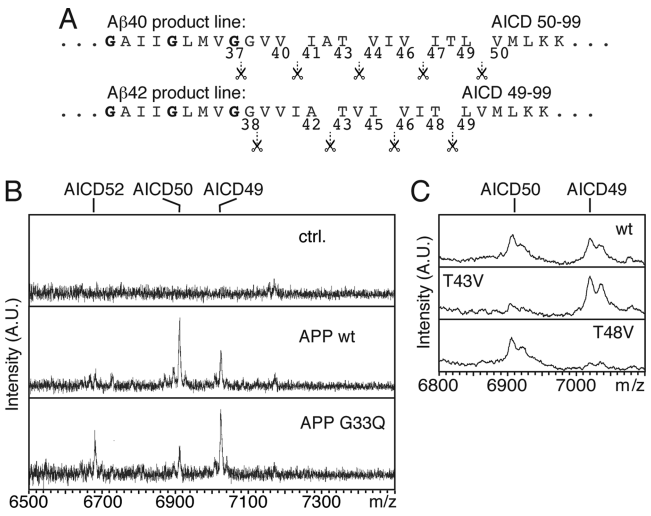

Figure 4. Impact of APP-TMS mutations on ε-cleavage. (A) Illustration of the two major Aβ-product lines generated through sequential processing of the APP TMS by γ-secretase. AICD50 corresponds to Aβ40 and AICD49 corresponds to Aβ42. (B) MALDI-MS spectra of AICD49 and AICD50 generated from APP wt and G33Q acquired using the reflector-detection mode; representative spectra from three independent experiments. (C) MALDI-MS spectra of AICD49 and AICD50 generated from APP threonine mutants acquired with the lower-resolution linear detection mode; representative spectra from two independent experiments. A.U.: arbitrary units; m/z: mass per charge; Ctrl.: control.

AICD49, while the cut between 49 and 50 results in Aβ40 and AICD50.[12−14,34] As depicted above, the Aβ levels measured from APP mutants G33Q and G33R as well as of T43V and T48V imply that a shift in Aβ product lines and thus presumably of the ε-cut had occurred. To identify shifts in product lines, we analyzed AICDs by mass spectrometry. To this end, we performed γ-secretase *in vitro* assays from cell lines expressing APP WT, G33Q, T43V, or T48V and enriched AICD by immunoprecipitation. MALDI-MS spectra of APP WT showed a strong signal for AICD50-99 and a lower signal for AICD49-99 corresponding well with high Aβ40 levels and low amounts of Aβ42 (Figure 4B,C). However, the mutant G33Q generated mainly the AICD49-99 and lower amounts of AICD50-99 corresponding to low levels of Aβ40 and high Aβ42 amounts as determined by ELISA (Figure 4B). In addition, AICD52-99 was detected that theoretically corresponds to the Aβ42 product line.[12] Likewise, the threonine mutant T43V generated predominantly AICD49-99, which corresponds to the Aβ42 product line and is in agreement with high Aβ42 levels detected by ELISA (Figure 4C). T48V generated predominantly AICD50-99, the initial cleavage site belonging to the Aβ40 product line. In conclusion, our MALDI-MS analysis shows that the production of AICD fragments is in agreement with the production of Aβ peptides observed for APP G33Q, T43V and T48V, and allows us to conclude that these substitutions indeed produce a shift in the ε-cut by γ-secretase and affect subsequent generation of Aβ40 and Aβ42.

DISCUSSION

We investigated three hydrophilic residues within the APP TMS and analyzed the impact of substitutions on Aβ generation. We first performed a comprehensive mutational analysis of residue glycine G33 located in the middle of the APP transmembrane-glycine zipper. Two predominant groups of mutations that differentially impact on Aβ generation were observed: (A) a group of hydrophilic/polar amino acids facilitating Aβ40 and Aβ42 generation and (B) a group of hydrophobic amino acids hindering Aβ40 and Aβ42 generation. This separation indicates that Aβ generation depends on the hydrophobicity of the amino acid side chain in position 33 and could be explained mechanistically by one of the three following theories: (I) The glycine residue is part of a glycine zipper motif, also named GxxxG motif, that was previously found to promote APP-TMS oligomerization.[35−37] Changing the hydrophobicity of the amino acid in position G33 would influence the strength of TMS assembly and in turn impact on Aβ40 and Aβ42 generation by affecting processing through γ-secretase. (II) It has been postulated that γ-secretase contains a substrate-binding site in addition to the catalytically active site.[38−43] The polar or hydrophobic nature of the amino acid at position G33 could alter the initial recognition of APP by γ-secretase affecting subsequent cleavage and finally production of Aβ40 and Aβ42. Indeed, polar side chains such as from N, D, and E can promote strong interactions within the hydrophobic environment of the lipid bilayer.[23] (III) The GxxxG sequence has been described to be a cholesterol-binding motif and APP was shown to sense cholesterol levels in the cell depending on the integrity of the GxxxG sequence.[44−46] It is therefore also possible that changes in the hydrophobicity at position G33 affect the cholesterol-binding ability of the APP TMS, and thus the Aβ peptide pattern is altered. It remains elusive whether one of these scenarios predominates or whether a combination of all three causes the different effects of polar and hydrophobic amino acids on Aβ generation.

In light of the above-mentioned scenarios, we would like to highlight the two amino acids lysine (K) and arginine (R) on Aβ40 production. Both amino acids have a bulky linear side chain ending with basic amino groups and are thus expected to behave similarly in membrane environments.[47] We found that G33K was processed similarly to the wild type with only a modest 12% decrease in Aβ40 and no effect on Aβ42 levels. Although lysine differs significantly from glycine, lysine is well tolerated in this position. In contrast, arginine almost completely prevents Aβ40 formation, whereas Aβ42 levels are again only modestly decreased by 12%. Thus, although lysine and arginine have similar side chains, we unexpectedly observed differential effects only on Aβ40 generation. This effect cannot be sufficiently explained by a single scenario mentioned above, suggesting that substrate recognition and cleavage by γ-secretase is a complex process modulated by various factors.

The threonine residues T43 and T48, which were subject of the second part of our mutational study are located in the C-terminal half of the APP TMS. In fact, the N-terminal half of the APP TMS has greater backbone dynamics than its C-terminal part, which is considered as more rigid, and these two regions are separated by a double-glycine hinge in the middle of the TMS.[48,49] Familial mutations of the APP TMS leading to early onset AD cluster in the C-terminal region between amino acids T43 and L52 implying that the structural integrity of this region is crucial for Aβ generation. Exchange of APP TMS's

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threonine residues to valine results in increased flexibility of this C-terminal region.[50] The authors suggest that enhanced conformational flexibility may change the pattern of γ-secretase processing.[50] We herein exchanged the same two threonine residues to valines and indeed found strong effects on Aβ generation. However, in our test system, the two mutants showed opposite effects: T43V enhanced Aβ42 generation, whereas T48 decreased Aβ42 levels. Previous studies following different objectives replaced the threonine residues against other amino acids. Exchange of T43 against hydrophilic amino acids decreased Aβ42 levels, while the exchange toward hydrophobic amino acids increased Aβ42 generation, in line with our observations using T43V.[51−53]

All these observations suggest that not only hydrophobicity or conformational flexibility is sufficient to explain the effects. One potential explanation could be that again the interaction with γ-secretase is differently affected by each threonine residue since they reside on opposite sides of the APP-TMS helix.

In summary, our set of APP-TMS mutations revealed that hydrophilic amino acid residues specifically impact Aβ generation. The interactions of G33, T43, or T48 with either the lipid environment, the γ-secretase complex or within a dimer may determine the predominant Aβ product line and Aβ species.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AD, Alzheimer's disease; AICD, APP intracellular domain; APP, amyloid precursor protein; Aβ, amyloid-beta; β-CTF, beta-C-terminal fragment; ELISA, enzyme-linked immunosorbent assay; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; SP-C99, signal peptide with C-terminal 99 amino acids of APP; TMS, transmembrane sequence

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