Aberrant Amyloid Precursor Protein (APP) Processing in Hereditary Forms of Alzheimer Disease Caused by APP Familial Alzheimer Disease Mutations Can Be Rescued by Mutations in the APP GxxxD Motif

Lisa-Marie Munter, Anne Botev, Luise Richter, Peter W. Hildebrand, Veit Althoff, Christoph Weise, Daniela Kaden, and Gerd Multhaup

From the Institut für Chemie und Biochemie, Freie Universität Berlin, Thielallee 63, 14195 Berlin, Germany and the Institut für Medizinische Physik und Biophysik, Charité, 10117 Berlin, Germany

The identification of hereditary familial Alzheimer disease (FAD) mutations in the amyloid precursor protein (APP) and presenilin-1 (PS1) corroborated the causative role of amyloid-β peptides with 42 amino acid residues (Aβ42) in the pathogenesis of AD. Although most FAD mutations are known to increase Aβ42 levels, mutations within the APP GxxxD motif are known to lower Aβ42 levels by attenuating transmembrane sequence dimerization. Here, we show that aberrant Aβ42 levels of FAD mutations can be rescued by GxxxD mutations. The combination of the APP-GxxxD mutation G33A with APP-FAD mutations yielded a constant 60% decrease of Aβ42 levels and a concomitant 3-fold increase of Aβ38 levels compared with the Gly33 wild-type as determined by ELISA. In the presence of PS1-FAD mutations, the effects of G33A were attenuated, apparently attributable to a different mechanism of PS1-FAD mutants compared with APP-FAD mutants. Our results contribute to a general understanding of the mechanism how APP is processed by the γ-secretase module and strongly emphasize the potential of the GxxxD motif in the prevention of sporadic AD as well as FAD.

APP2 and APLPs were conventionally thought to exist and to act as monomers. However, biochemical and structural data have accumulated over the past few years, indicating that APP and APLPs exist as functional dimers or even are present in higher oligomeric units (1–6). Interactions of APP and APLPs were reported to promote cell adhesion in a homo- and heterotypic manner (7, 8). Among other mechanisms, the varying strength of APP dimerization mediated through N-terminal sites (5) or by the transmembrane sequence (TMS) (9) has been reported to influence APP processing.

APP is first cleaved by the β-site APP cleaving enzyme and is then sequentially processed by the γ-secretase complex to generate Aβ peptides of varying length (10, 11). γ-Secretase cleavage specificity is modulated by the GxxxD (“G-triple-x-G”) dimerization motif of the APP-TMS, and we showed previously that APP can be cleaved as a homodimer by β- and γ-secretases (9). APP, APLP1, and APLP2 share similar interaction motifs and can form APP-APLP1 and APP-APLP2 complexes (7). Co-transfections of APP with APLP1 or APLP2 influenced APP processing into Aβ leading to decreased Aβ40 and Aβ42 levels likely through an influence on γ-secretase cleavages (7).

According to the amyloid hypothesis, Aβ peptides represent the main culprit of Alzheimer disease (AD). Based on this assumption is the appealing prediction that reducing Aβ levels would ameliorate Alzheimer symptoms (12, 13). In the current model of Aβ generation, the initial cut at the e-site is executed by the presenilins of the γ-secretase complex, leading to formation of the APP intracellular domain and Aβ40 or Aβ48 peptides (10, 14). The latter two likely remain bound to the active site and are successively cleaved every three to four residues at the ε-site and at the γ-sites (11, 15). Most likely, two product lines exist. In the product line encompassing Aβ40, Aβ49 is trimmed to Aβ40, and in the other product line, Aβ48 is the precursor of Aβ42 and Aβ38 (10, 15). When we studied homodimerization of APP-TMS, a bacterial test system revealed that glycine residues 29 and 33 of the GxxxD amino acid motif represent the major helix-helix contact site (see Fig. 1A). Substitutions of either of the two glycine residues not only diminished APP-TMS homodimer stability but also drastically decreased Aβ42 and increased Aβ38 levels, among other changes (9). Our findings that the γ-secretase complex can cleave dimeric substrates complement the current model of successive cleavages producing Aβ peptides from two predominant product lines (15).

Importantly, mutations in three different genes are associated with AD and were described to increase the ratio of Aβ42 to Aβ40 in mice and humans (16–18). Early onset dominant...
AD was first associated with the APP gene and found to alter the amount of Aβ produced and/or the ratio of Aβ42 to Aβ40 (16, 19). Subsequently, early onset familial mutations in the presenilins were also linked to AD (17, 20). Using some of these mutations, we further investigated the GaαG-mediated effects on γ-secretase cleavage by combining GxxxG mutations with FAD mutations.

We found here a constant 60% decrease in Aβ42 and a 3-fold increase in Aβ38 levels for APP-FAD mutants when we impaired the APP-GxxxG-mediated interaction by introducing the mutation G33A. However, G33A only had mild effects in the presence of PS1-FAD mutations. Furthermore, we found that the analyzed FAD mutants can be divided into subgroups: (i) APP-FAD mutants that increase Aβ42 and Aβ38 levels and either show a concomitant increase or a decrease of Aβ40 levels, (ii) APP-FAD mutants that do not affect Aβ42 and Aβ38 levels but exclusively decrease Aβ40 levels, and (iii) PS1-FAD mutants that increase Aβ42, but decrease Aβ38 levels.

**EXPERIMENTAL PROCEDURES**

*Plasmids and Transfections*—The plasmid pCEP4 containing the coding sequence for APP695 with an N-terminal Myc tag and C-terminal FLAG tag was used as a template to introduce the APP-FAD mutations and G33A by site-directed mutagenesis. Either G33A or the FAD variants were used to generate the double mutant constructs APP-FAD-G33A in a second site-directed mutagenesis. The same method was applied to generate SPA4CT-G33A from pCEP4-SPA4CT with a C-terminal FLAG tag and the PS1-FAD mutants from pcDNA3.1/zeo and MALDI-MS—SH-SY5Y cells routinely were cultured as described (9). Aβ40/Aβ42 ELISAs were performed according to the manufacturer’s instructions (The Genetics Company). The following antibody combinations were used: for Aβ38, BA1–13 (Covance/Signet) and W0-2-biotin (TGC); for sAPPα, anti-Myc antibody (Cell Signaling Technology) and W0-2-biotin; and for total Aβ, W0-2 or 4G8 (Covance) and 4G8-horseradish peroxidase (Covance) or W0-2-biotin, respectively. W0-2-biotin was recognized by streptavidin-conjugated horseradish peroxidase, and ELISAs were developed with 1-Step Ultra-TMB (Pierce) and were measured at 450 nm in a microplate reader (Anthos). Western blot analyses and immunoprecipitations were performed as published (9). Briefly, 250 μl of conditioned media were diluted with phosphate-buffered saline and precipitated with polyclonal rabbit antibody 18-1 (generated to Aβ1-40) or W0-2 and protein A- or G-Sepharose (GE Healthcare), respectively. For Western blot analysis, cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and immunostained. For MALDI-MS analyses, Aβ was eluted twice from Sepharose with 500 μl 50% acetic acid, and the eluate was vacuum-dried. MALDI-MS analysis was carried out as described previously (9).

**Structural Model**—The APP-TMS model from previous analyses based on the tertiary structure of glycophorin A was used as a template (9). The FAD mutants were added to the structure using the Swiss-PdbViewer. The most likely rotamers were selected before the model was energetically minimized
applying the GROMACS Software. All figures were produced with the PyMOL Molecular Graphics System.

RESULTS

Analysis of APP-FAD Mutants—We analyzed APP processing of nine APP-FAD mutations scattered around the three secretase cleavage sites, i.e., K670N/M671L at the β-site (Swedish, hereafter referred to as “sw”), A692G (Flemish) and E693G (Arctic) near the α-site, and T714I (Austrian), V715M (French), I716V (Florida), V717G, V717F, and L723P (Australian) at the γ-secretase cleavage sites (Fig. 1A) (19, 21–28). The APP-FAD mutants were stably expressed in SH-SY5Y cells and yielded 100% (mean ± S.E., n = 7–16) total Aβ levels (APP-wt set as 100%, see supplemental Fig. 5A and (mean ± S.E., n = 4–12), F, Aβ40 levels, APP-wt set as 100% (mean ± S.E., n = 7–16). A–E, asterisks indicate significant differences to APP-wt (*, p < 0.01, **, p < 0.001, one-way ANOVA type Dunnett). Shaded bars indicate mutations increasing Aβ42 and Aβ38. Horizontal shaded bars highlight mutations only decreasing Aβ40. Horizontal lines mark the fragment levels of APP-wt for better comparison with the mutants.

FIGURE 2. Analysis of APP-FAD mutations. Quantification of secreted sAPPα, total Aβ, Aβ42, Aβ40, and Aβ38. A, sAPPα levels, APP-wt (without FAD) set as 100% (mean ± S.E., n = 5–16). B, total secreted Aβ levels, APP-wt set as 100% (mean ± S.E., n = 6–14). Note that A692G and E693G could not be analyzed by total Aβ ELISA as the FAD mutations alter the epitope of the antibody 4G8. C, total Aβ levels of A692G and E693G were analyzed by immunoprecipitation with antibody 18–1 and Western blot analysis with W0–2. D, Aβ42 levels, APP-wt set as 100% (mean ± S.E., n = 5–17). E, Aβ38 levels, APP-G33A set as 100%, see supplemental Fig. 5B and (mean ± S.E., n = 7–16). F, Aβ40 levels, APP-wt set as 100% (mean ± S.E., n = 4–12), A–E, asterisks indicate significant differences to APP-wt (*, p < 0.01, **, p < 0.001, one-way ANOVA type Dunnett). Shaded bars indicate mutations increasing Aβ42 and Aβ38. Horizontal shaded bars highlight mutations only decreasing Aβ40. Horizontal lines mark the fragment levels of APP-wt for better comparison with the mutants.

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Effect of the GxxxG Motif on APP-FAD Processing—To investigate the role of the GxxxG motif known to mediate APP-TMS dimerization, we tested constructs encoding individual APP-FAD mutations in combination with the GxxxG mutation G33A (APP-FAD-G33A). All APP-FAD-G33A-derived constructs generated sAPPα and total Aβ levels similar to the respective APP-FAD variant alone, indicating that their processing is equally efficient as that of APP-wt or APP-FAD alone (supplemental Fig. S1). However, the striking difference is that the APP-FAD-G33A constructs drastically reduced Aβ42 levels compared with APP-FAD alone (Fig. 3A). Mutants A692G/G33A, T714I/G33A, and V715M/G33A yielded Aβ42 levels similar to APP-wt. The mutants E693G, I716V, V717G, V717F, and L723P in combination with G33A did not even reach Aβ42 levels of APP-wt. However, the APP-sw-G33A mutant still produced 1.6-fold higher Aβ42 levels than APP-wt (Fig. 3A). For better comparison and to calculate the percent impact of G33A on processing, data of Gly33-wt were each normalized to 100% (mean ± S.E., n = 5–17). For better comparison, bars of APP-FAD mutations as in Fig. 2D are shown (G33-wt, gray bars). The mutation G33A leads to a relative decrease in Aβ42 levels (black bars). Asterisks indicate significant differences to APP-wt (*, p < 0.01; **, p < 0.001, one-way ANOVA type Dunnett). The horizontal line marks Aβ42 level of APP-wt for better comparison. Original data of sAPPα, total Aβ, and Aβ40 ELISA are shown in supplemental Fig. S1. B–D, Aβ levels of the G33A constructs (black bars) normalized to the respective Gly33-wt constructs, which were set as 100% (mean ± S.E., n = 5–17). D, relative Aβ38 levels (mean ± S.E., n = 4–12). B–D, horizontal lines mark the fragment levels of APP-G33A for better comparison with the FAD mutants. E, analysis of expression levels of SPA4CT-related constructs in SH-SY5Y cells. The fragments show comparable expression levels, although these are generally lower than for SPA4CT-wt. Western blot was stained with monoclonal W0-2. F, levels of secreted Aβ from SPA4-fragments are normalized to SPA4CT-wt, which was set as 100% (mean ± S.E., n = 3–9). The mutation G33A has no impact on Aβ40 levels but decreases Aβ42 and increases Aβ38 levels. w/o, without.
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sequences tested, the G33A mutation reduced Aβ42 levels by ~60% (Fig. 3C) and a 3-fold increase of Aβ38 levels (Fig. 3D), whereas Aβ40 levels remained unchanged (Fig. 3B). Thus, the G33A mutation within the GxxxG motif possesses the same strong impact on APP-FAD mutations as on APP-wt in terms of decreasing Aβ42 production and thus has a FAD-rescuing effect. By MALD-I-MS analyses, we confirmed that no construct generated an aberrant pattern of Aβ species (supplemental Fig. S2). This data indicates that single amino acid changes within the GxxxG motif can rescue APP-FAD-related effects.

This observation raised the question of whether G33A affects the processing at early (ε-site) or subsequent (γ-sites) cleavage steps of γ-secretase. Therefore, two extended constructs were generated encoding elongated Aβ species that were shown to be either processed as precursors of the Aβ42 line, i.e. Aβ(1–51), or by the Aβ40 line, i.e. Aβ(1–52) (29). As these constructs were generated from the C-terminal 99 residues of APP (SPA4/CT) (30), the fragments are named SPA4/1–51 and SPA4/1–52. The constructs are normally expressed in SH-SY5Y cells and processed like the C-terminal fragment of APP (Fig. 3E). The quantification of Aβ levels shows that SPA4/1–52 predominantly generates Aβ40 but neither Aβ42 nor Aβ38 (Fig. 3F). SPA4/1–51 generates mainly Aβ42 and Aβ38. SPA4/1–51 containing the mutation G33A shows 2.5-fold reduced Aβ42 levels and 2-fold increased Aβ38 levels compared with Gly33-wt. Aβ40 levels are not affected in both constructs. Thus, the mutation G33A similarly influences processing of SPA4/1–51, SPA4/1–52, APP-wt, and APP-FAD. This indicates that the mutation G33A has a universal effect on Aβ42 and Aβ38 generation. Importantly, the result also shows that the allocation to one or the other product line cannot be modulated by G33A mutation.

Analysis of PS1-FAD Mutants—Three mutations, i.e. G378A, L381A, and G384A, located near or within the highly conserved GXGD motif of PS1 in close proximity to the catalytically critical aspartate residue 385 within TMS-7 (31–33) were analyzed (Fig. 1C). As substrate, we used SPA4/CT instead of the full-length APP, as SPA4/CT yields higher Aβ levels (9). Stably expressing SH-SY5Y cell lines were selected for expression of similar levels of holo-PS1, the N-terminal fragment of PS1, and SPA4/CT by Western blot analysis (supplemental Fig. S3). Efficiency of SPA4/CT processing by PS1-FAD mutants did not vary between cell lines as indicated by total Aβ levels measured (Fig. 4A). All three PS1-FAD mutants led to increased levels of Aβ42 and decreased levels of Aβ38 (Fig. 4, C and D). Aβ40 levels were found specifically reduced for PS1-G33A (Fig. 4B), a mutant that has already been described to selectively lower Aβ40 production (34). Thus, an obvious difference between the investigated PS1-FAD and APP-FAD mutations are the consistently increased Aβ42 levels of PS1-FAD at the expense of decreased Aβ38 levels, whereas APP-FAD mutants that increase Aβ42 also increase Aβ38 levels. This also indicates that PS1-FAD processing is divergent from APP-FAD.

Impact of the GxxxG Mutation G33A on PS1-FAD Processing—Effects of the GxxxG motif on APP and SPA4/CT processing are very similar, as described previously (9). Now, to investigate possible GxxxG-mediated influences on PS1-FAD mutants, we stably coexpressed SPA4/CT-G33A and PS1-FAD mutants in SH-SY5Y cells and determined levels of Aβ in the cell culture supernatants by ELISA (Fig. 5 and supplemental Fig. S4). Aβ levels generated from Gly33-wt substrates were set as 100%. Total Aβ levels were similar for all constructs tested indicating that the SPA4/CT-G33A protein was efficiently processed by the respective PS1-FAD γ-secretase complex (Fig. 5A). As expected for the G33A mutant, Aβ40
levels were not affected (Fig. 5B). However, the G33A mutation showed the tendency to decrease Aβ42 levels (Fig. 5C) and to increase Aβ38 levels; the latter at least holds true for the G387V and L381V mutations compared with Gly33-wt (Fig. 5D). Attributable to the close proximity to the active site the G384A mutation might follow a different mode of action. There were no aberrant patterns of Aβ species (supplemental Fig. S5). Although the effect of G33A on Aβ42 and Aβ38 levels in the presence of PS1-FAD mutations was visible, it was not as strong as observed for the APP-FAD mutations.

DISCUSSION

Processing of APP-FAD and PS1-FAD Mutants—Here, we describe a previously unrecognized relationship between mutations in juxtamembrane positions and γ-secretase cleavages, although the mutations are located ~20–40 amino acid residues apart from the γ-secretase sites. This is exemplified by the mixed effect found on Aβ species for the APP-sw mutant showing a 4-fold increase in Aβ42 and Aβ38 levels but an only 2-fold increase Aβ40 levels. So far, influences on Aβ42/Aβ40 ratios by APP-sw were only described to vary with the age of mice expressing APP-sw (35) or APP-sw and V717F (36).

We also observed that most of the APP-FAD mutations either increase Aβ42 and Aβ38 levels or solely decrease Aβ40 levels. We explain this observation with the sequential cleavage model of γ-secretase proposing the existence of two product lines (9, 15), the Aβ40 line and the Aβ42 line (Fig. 6A). We suggest that APP-FAD mutations cause a general shift away from the Aβ40 line toward the Aβ42 line, explaining why Aβ40 levels were found decreased and both Aβ42 as well as Aβ38 levels were increased (Fig. 6A). Interestingly, molecular modeling reveals that the amino acid side chains of APP-FAD mutations stick out of the dimer interface and thus affect APP-FAD-TMS dimerization in a cellular environment (Fig. 6, A and B). However, Gorman et al. (37) observed an impact of FAD mutations on oligomerization of synthetic peptides in micelles. From our data, we speculate that APP-FAD mutations, especially those located in the C-terminal region, may shift the affiliation to the Aβ42 product line by affecting enzyme-substrate recognition rather than that they impair TMS dimerization itself. Thus, the initial recognition of APP-FAD mutations seems to decide on the pathway followed (Aβ42 or Aβ40 line). This view is supported by results describing that APP-FAD mutations in the C-terminal region affect ε-cleavage, leading to an increase of APP intracellular domain levels (APP intracellular domain) promoting the Aβ42 line (38, 39). Although the α-helices likely need to be unfolded prior to γ-secretase cleavages, it might be of interest that the peptide bonds cleaved in the Aβ42 line reside outside the dimer, whereas the peptide bonds of the Aβ40 line reside within the dimer interface.

For the PS1-FAD mutants analyzed, a reduction in Aβ38 but an increase in Aβ42 levels was the major change observed, indicating that PS1-FAD mutations generally act differently from
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APP-FAD mutations. In agreement with the sequential cleavage model, PS1-FAD mutations might lead to an inhibition of flux through the Aβ42 pathway, which could account for the decrease of Aβ38 and increase of Aβ42 levels (Fig. 6A). The inhibition of cleavage flux is in agreement with the loss-of-function hypothesis of PS1-FAD mutations (40). The apparently different APP-FAD and PS1-FAD mechanisms question cell culture or mouse models where these mutations are combined to accelerate the Aβ production and pathology.

Impact of the GxxG Motif—We have reported previously that the sequence motif GxxG within the APP-TMS has a regulatory impact on the Aβ species produced. The mutation G33A attenuated the TMS dimerization strength by 20%, specifically reduced the formation of Aβ42 by 60%, left Aβ40 levels unaffected, but increased the formation of Aβ38 (3-fold) and shorter Aβ species (9). When we analyzed the GxxxG motif in combination with APP-FAD mutations, we found that the G33A mutant yielded the same shift, i.e. a 60% decrease of Aβ42 levels and a concomitant 3-fold increase of Aβ38 levels for all APP-FAD mutants. Thus, G33A in combination with APP-FAD mutations affected γ-secretase processing in the same way as when combined with APP-wt. Furthermore, in protein constructs being degraded in a predetermined product line (SPA4/1–51, SPA4/1–52) G33A had the same strong effect on the consecutive processing. This implies that G33A particularly affects γ-cleavages rather than the primary ε-cleavage step. This also indicates that G33A exclusively influences processing within the Aβ42 line.

In the presence of PS1-FAD mutations, the impact of G33A on Aβ generation was diminished, which might be attributable to the possible inhibition of substrate flux by PS1-FAD mutations. Thus, the mutation G33A acts downstream of APP-FAD mutations but only partially downstream of PS1-FAD mutations.

In addition to this data, a product-precursor relationship of Aβ42 and Aβ38 was indicated by the effects on Aβ production by (i) nonsteroidal anti-inflammatory drugs or γ-secretase modulators (41), (ii) several γ-secretase inhibitors (42), (iii) N-terminal elongation of pen-2 (43), and (iv) GxxG mutations (9) as well as direct detection (V) of the tetrapeptide V39V1A42 arising from the Aβ42 cleavage that generates Aβ38 (15). Page et al. (34) and Czirr et al. (44) concluded from their work that Aβ42 and Aβ38 are not related in their production as in the presence of PS1-FAD mutants nonsteroidal anti-inflammatory drugs sulindac sulfide and fenofibrate only had an attenuated effect on Aβ38 and Aβ42 levels. In agreement with this, we found only a tendency of G33A to change the Aβ38/Aβ42 levels in the presence of PS1-FAD mutants. We assumed that the attenuated effects are attributable to the inhibition of substrate flux by PS1-FAD mutations. Concordantly, we suggested previously that nonsteroidal anti-inflammatory drugs might act by modulating the substrate dimer stability (9), which recently has been supported by the finding that nonsteroidal anti-inflammatory drugs are substrate-targeted modulators, which possibly bind to the Aβ sequence (45).

CONCLUSION

APP-FAD and PS1-FAD mutations act differently on Aβ42/Aβ40 production. Mechanistically, the analyzed familial mutations can be divided into three subgroups: (i) APP-FAD mutations that increase Aβ42 and Aβ38, (ii) APP-FAD mutations that decrease Aβ40, and (iii) PS1-FAD mutations that increase Aβ42, but decrease Aβ38 levels. In the early steps of APP processing, APP-FAD mutations decide on the affiliation to the one or the other product line, and subsequently, the mutation G33A affects processing within the Aβ42 line.

An impairment of the GxxxG-mediated dimerization of APP was sufficient to reduce the pathological processing effects of APP-FAD mutants. On average, 60% less Aβ42 and 3-fold more Aβ38 were produced by the APP-FAD-G33A mutants. The effects of G33A were found attenuated with PS1-FAD mutants attributable to the different pathogenic mechanism of PS1-FAD. Thus, our data supports the idea that the APP GxxxG motif represents a new drug target site not only for sporadic AD but also for early onset FAD.

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