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Homodimerization Protects the Amyloid Precursor Protein C99 Fragment from Cleavage by γ-Secretase

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Supporting Information

ABSTRACT: The amyloid precursor protein (APP) is a single-span integral membrane protein whose C-terminal fragment C99 is cleaved within the transmembrane helix by γ-secretase. Cleavage produces various Aβ peptides that are linked to the etiology of Alzheimer's disease. The transmembrane helix is known to homodimerize in a sequence-specific manner, and considerable controversy about whether the homodimeric form of C99 is cleaved by γ-secretase exists. Here, we generated various covalent C99 homodimers via cross-linking at engineered cysteine residues. None of the homodimers was cleaved *in vitro* by purified γ-secretase, strongly suggesting that homodimerization protects C99 from cleavage.

The amyloid precursor protein (APP) is an N_{out} single-span membrane protein whose sequential cleavage by β- and γ-secretase within the amyloidogenic pathway leads to the formation of Aβ peptides. Shedding by β-secretase produces the C99 protein; γ-secretase makes initial endoproteolytic cleavages close to the C-terminus of the C99 transmembrane domain (TMD), thus producing the APP intracellular domain (AICD) and long Aβ peptides. Processive cleavage toward the N-terminus within Aβ produces a series of differently sized Aβ peptides, including Aβ42 and Aβ40.[1] Aβ peptides form toxic protofibrillar aggregates and amyloid plaques that are thought to lead to Alzheimer's disease.[2,3]

Apart from C99, γ-secretase cleaves ∼90 other single-span membrane proteins.[4] The quaternary structure of most substrates is unknown. TMD-mediated homodimerization has been shown for C99,[5−7] Notch,[8] ErbB4,[9] and several phosphatases.[10] In the case of C99, TMD-mediated homodimerization was confirmed by nuclear magnetic resonance (NMR) spectroscopy. Accordingly, Gly33 and Gly37 being part of a tandem GxxxG motif within its TMD helix are central to the helix−helix interface[11−13] that is supported by molecular modeling.[14,15] A GxxxG motif frequently forms the interface of homodimerizing TMDs.[16] Other studies suggest different interfaces.[17−19] These structural differences may be reconciled by the ability of the dimer to adopt alternative structures as recently suggested by a systematic modeling study.[20]

The potential biological relevance of C99 homodimerization has been debated controversially. Some reports promote a role of dimerization in cleavage by γ-secretase. For example, homodimerization of APP via a cysteine in place of Lys28 (K28C) located at the TMD N-terminus was reported to augment total Aβ peptide production ∼7-fold.[21] In addition, Aβ42, but not Aβ40, production in cultured cells was facilitated by covalent homodimerization through an artificial disulfide bridge introduced at position 17.[5] In line with a role of homomerization in cleavage, an increased substrate concentration led to a higher ratio of Aβ40 to Aβ42 peptides.[22] Further, point mutant G33I and double mutant G29A/G33A within the TMD GxxxG motif weakened homodimerization and in parallel decreased Aβ40 and Aβ42 production in favor of Aβ37 and Aβ38 formation in cultured cells.[5,23] This correlation between homodimerization and Aβ production was interpreted by a model in which a homodimeric substrate exists within the catalytic cavity of γ-secretase; in this model, weakened self-interaction would facilitate processive γ-secretase cleavage, thus favoring production of the shorter Aβ38.[5] Finally, γ-secretase was reported to simultaneously cleave multiple C99 molecules, i.e., dimeric or oligomeric forms of C99, in one catalytic turnover.[24]

Other studies challenge the proposition that C99 is cleaved as a homodimer. The introduction of a G29L/G33L double mutation stabilized the TMD−TMD interface and concomitantly reduced Aβ40 and Aβ42 production without inhibiting initial cleavage.[14] The interpretation of this result is not unambiguous, however, because the mutant appeared to adopt a TMD−TMD interface different from that of the wild-type (wt) protein.[14] Another approach used regulated homodimerization through FK506-mediated homodimerization of the FKBP domain fused to the C99 C-terminus. In that study, dimerization, however incomplete (70%), resulted in a 50% drop in Aβ production, suggesting an inhibition of cleavage by dimerization.[25] Most recently, noncovalent C99 dimers and trimers eluted from sodium dodecyl sulfate (SDS) gels were subjected to γ-secretase cleavage *in vitro*.[26] That study revealed strongly reduced levels of Aβ formation when the dimers or trimers were used as the substrate, again suggesting inhibition of cleavage by oligomerization. However, the question of whether the low level of Aβ peptides reflected merely inefficient

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cleavage of dimers or trimers or cleavage of monomeric C99 formed by partial oligomer dissociation after elution remained unresolved. In addition, this study did not control for the structural integrity of the protein eluted from the gel that might have lost its original secondary structure.[26]

Here, we decided to investigate *in vitro* cleavage of covalent C99 homodimers. We find that the extent of cleavage was strongly reduced and no disulfide-linked Aβ or AICD products could be identified. Thus, our results demonstrate that the homodimers are not cleaved by γ-secretase.

We designed four cysteine mutants of C99 for cross-linking: S8C (numbering begins from D1 of C99), a conservative mutation far from the N-terminus of the TMD; L17C, whose homodimer was investigated previously in eukaryotic cells;[5] S26C, located within the unstructured turn preceding the TMD;[27] and S59C, situated downstream from the TMD C-terminus (Figure 1A). To leave the natural TMD−TMD interface undisturbed, no cysteine was introduced into the TMD.

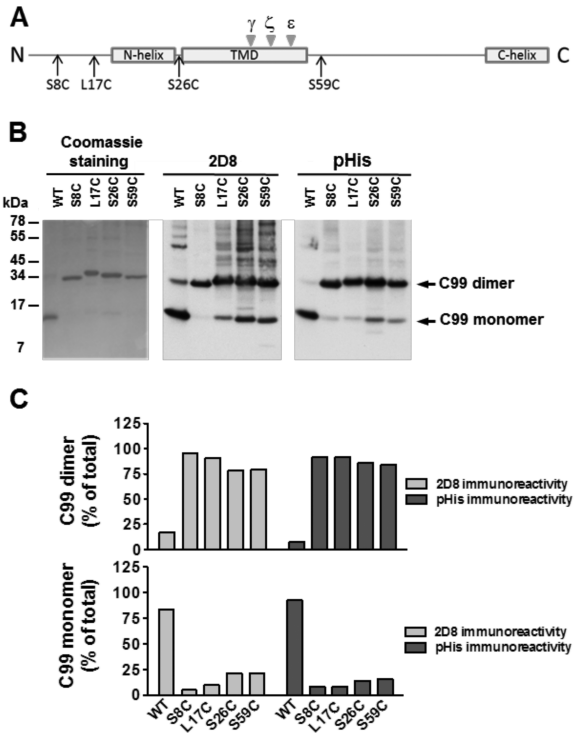

Figure 1. Homodimer formation of C99 cysteine mutants. (A) Location of cysteine mutations within C99. The principal cleavage sites are given above the TMD. (B) Assessing the extent of covalent homodimerization of mutant C99 by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) followed by Coomassie staining (left) or Western blotting and staining with the 2D8 antibody directed against Aβ peptides (center) or an antibody recognizing the His_6 tag (right). (C) Densitometric evaluation of the blots shown in panel B.

All mutants as well as wt C99 were expressed in *Escherichia coli*. After cell lysis in the presence of SDS, the mutants were covalently homodimerized by Cys oxidation using the Cu^{2+}/*o*-phenanthroline complex.[28] The His_6-tagged proteins were then purified to homogeneity by metal chelate chromatography. The Coomassie blue-stained SDS gel shown in Figure 1B (left panel) shows that the cysteine mutants are quantitatively cross-linked to homodimers with only minor traces of remaining monomer. Densitometry of corresponding immunoblots stained with either antibody 2D8 directed against the N-terminus of C99 or an antibody recognizing the His_6 tag identified ∼80−90% homodimer (Figure 1B,C, central and right panels); the stronger visibility of the monomer is attributed to the higher sensitivity of immunoblotting. wt C99 exhibited ∼10−20% noncovalent homodimer in immunoblots as seen previously.[6,29]

The covalent homodimers were compared to wt C99 to test their structural integrity. First, sucrose velocity gradient centrifugation was performed to exclude the possibility that cross-linking produces nonspecific high-molecular weight aggregates. Because of the low resolution of sucrose gradients, C99 monomers and dimers appear to overlap with a slight difference in migration that is consistent with the mainly monomeric nature of the wt and the homodimeric structure of the disulfide-linked Cys mutants (Figure S2A). Importantly, no significant amounts of high-molecular weight forms were found at the bottoms of the gradients, indicating that covalent homodimerization did not induce unspecific aggregation. Second, the secondary structures of wt C99 and covalent homodimers were compared by circular dichroism (CD) spectroscopy (Figure S2B). Deconvoluting the CD spectra of wt, L17C, S26C, and S59C into individual spectral components revealed that the proteins contain similar amounts of α-helix (∼20%) and significant random coil and various β-conformations (Figure S2B, inset). The three helical domains of C99[27] account for ∼40% of all residues. Assuming a frayed N- and C-helical turn in each of these helices and partial unfolding at the central $Gly_{37}Gly_{38}$ hinge within the TMD[27,30] implies that only ∼20% of residues are in a true α-helical conformation, which is close to the value estimated here by CD spectroscopy. In contrast to that, S8C contains only ∼10% helix, which is offset by an increased content in β-structures (Figure S2B). The generally high contents in the β-conformation seen here are surprising given that mostly disordered structure was previously assigned to nonhelical parts of C99 by NMR spectroscopy.[11] A recent analysis by Fourier transform infrared spectroscopy, however, also identified β-sheet structure in a region upstream of the TMD.[13] Collectively, these controls demonstrate that the covalent homodimerization of C99 by disulfide bridging does not induce unspecific aggregation or loss of α-helical secondary structure, except for S8C with its reduced α-helicity.

To assess the impact of covalent homodimerization on cleavage by γ-secretase, wt C99 and the homodimers were compared in *in vitro* cleavage assays where SDS-solubilized C99 is added to purified γ-secretase in phosphatidylcholine liposomes. The substrate is present in the assay at a protein/lipid ratio of 1/1400, which is equivalent to a concentration of 0.0007 mol %. At this concentration, wt C99 is expected to exist as a monomer because the dissociation constant in a phosphatidylcholine/phosphatidylglycerol (3/1 molar ratio) membrane is 0.47 mol %.[7] Under nonreducing conditions, that is in the absence of dithiothreitol (DTT) (Figure 2A, "−DTT"), a significant part of the wt C99 control is cleaved into Aβ peptides (collectively recognized by the 2D8 antibody) and AICD (detected by the anti-His_6 antibody), as expected. Importantly, only little cleavage is observed with disulfide-bridged dimers as shown by the immunoblots (Figure 2A) and their quantification (Figure 2B). According to densitometric evaluation of several experiments, on average, only less than ∼10−30% of Aβ and AICD was produced from the mutants relative to wt C99. Of note, higher contents of the C99

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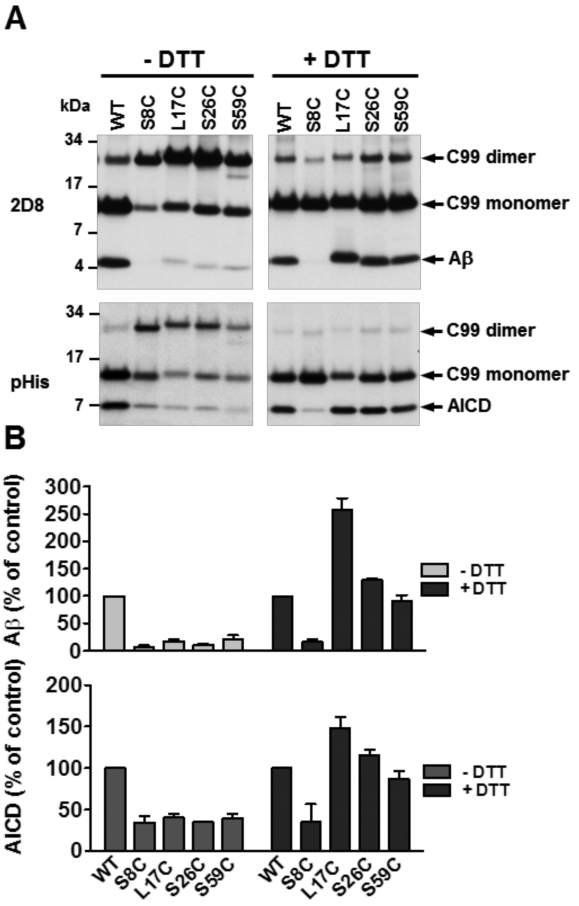

Figure 2. γ-Secretase cleavage assays. (A) Cleavage of monomeric wt C99 and the indicated cysteine mutant derivatives was assessed under nonreducing (−DTT) or reducing (+DTT) conditions. After overnight incubation, nonreducing SDS−PAGE sample buffer was added to the samples. These were then subjected to SDS−PAGE followed by immunoblot analysis using antibodies 2D8 to detect Aβ (top panels) or anti-penta-His (pHis) to detect AICD (bottom panels). Note that substantial substrate cleavage of dimeric C99 derivatives was only observed upon prior monomerization by DTT. (B) Quantification of three independent experiments by densitometric evaluation of the blots. Data represent the Aβ (top panel) and AICD (bottom panel) signals of the mutants relative to wt substrate (=100%). Bars denote the mean ± the standard error.

monomer were seen when mutant proteins were recovered from cleavage assays (Figure 2) than when pure proteins were recovered (Figure 1B). This may result from partial disulfide exchange between the disulfide-bridged substrates and free cysteine residues of γ-secretase. The minor amounts of Aβ and AICD produced from the mutants may thus result from partially monomerized substrate. However, it is clear that both Aβ and AICD do not result from cleavage of covalent homodimers as indicated by the absence of dimeric Aβ (which would be produced by cleavage of dimeric S8C, L17C, and S26C proteins) and by our inability to detect dimeric AICD (which would be produced from the S59C dimer). We cannot exclude the possibility that potentially formed dimeric AICD overlaps with C99. As a control, the assays were also performed after monomerization induced by DTT; DTT does not affect γ-secretase activity as demonstrated by similar cleavage efficiencies of wt C99. Indeed, under these conditions, the monomeric L17C, S26C, and S59C mutants were cleaved with efficiencies similar to that of wt C99 (Figure 2, "+DTT"). This control experiment verifies that the strongly reduced cleavability seen with the respective homodimers (Figure 2, "−DTT") does not result from mutating the primary structure. In the case of S8C, however, even monomerization by DTT treatment did not restore cleavability. The S8C mutation therefore appears to inhibit cleavage in a manner independent of dimerization. Possibly, the changed structure of this mutant (Figure S2B) is responsible for the inhibitory effect of the S8C mutation.

We conclude that a covalent C99 homodimer is not cleaved by γ-secretase. This finding contradicts earlier suggestions implying a multimeric substrate.[5,21,22,24] Our result is in line, however, with other studies in which the extent of cleavage decreased in response to artificial homodimerization in cultured cells[14,25] and the recent demonstration that noncovalent C99 dimers and trimers excised from an SDS gel are not cleaved *in vitro*.[26] Our current experiments document that no dimeric products are formed from covalent dimers. This supports the conclusion that homomerization protects the substrate from cleavage by γ-secretase. It is quite conceivable, however, that the demonstrated homodimerization of APP and C99 and the sequence-specific contribution of the TMD[5,11−15,17−19,21] support a function apart from cleavage by γ-secretase.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00986.

A detailed description of Materials and Methods and Figure S1 (PDF)

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Author Contributions

E.W. purified γ-secretase and performed cleavage assays. A.J. produced mutant C99 dimers and performed sucrose gradient centrifugation and CD spectroscopy. H.S. supervised cleavage assays and contributed to the writing of the paper. D.L. designed the project, supervised sucrose gradient centrifugation and CD spectroscopy, and wrote the paper. All authors analyzed the data and interpreted the results.

E.W. and A.J. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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