RESEARCH ARTICLE SUMMARY

STRUCTURAL BIOLOGY

Recognition of the amyloid precursor protein by human γ-secretase

Rui Zhou*, Guanghui Yang*, Xuefei Guo, Qiang Zhou, Jianlin Lei, Yigong Shi†

INTRODUCTION: Alzheimer's disease (AD) is characterized by amyloid plaques in the brains of patients. The primary components of amyloid plaques are β-amyloid peptides (Aβs), which are derived from the amyloid precursor protein (APP). APP is first cleaved by α- or β-secretase to generate an 83- or 99-residue transmembrane (TM) fragment (APP-C83 or APP-C99), respectively. APP-C99 is then cleaved by the intramembrane aspartyl protease γ-secretase to generate the peptides Aβ48, Aβ45, Aβ42, and Aβ38 or Aβ49, Aβ46, Aβ43, and Aβ40. Of these, Aβ42 and Aβ43 are particularly prone to aggregation and formation of the amyloid plaques. Another substrate of γ-secretase is the Notch receptor. Aβ oligomers may contribute to AD development. Therefore, inhibition of γ-secretase represents a potential therapeutic treatment for AD. Unfortunately, γ-secretase inhibitors caused severe side effects without any clear clinical benefits for AD patients, perhaps owing to their inhibition of Notch cleavage.

Human γ-secretase comprises four subunits: presenilin (PS), PEN-2, APH-1, and nicastrin. As the catalytic subunit of γ-secretase, presenilin has two isoforms (PS1 and PS2). PS1—and, to a lesser extent, PS2 and APP—are frequently targeted for mutations in familial AD patients. Although free γ-secretase has been structurally characterized, how it recognizes APP remains largely unknown.

RATIONALE: Structural comparison of APP and Notch recognition by γ-secretase may reveal differences that can be exploited toward the design of substrate-specific inhibitors. However, the γ-secretase–substrate complex is extremely transient and has defied all efforts of isolation for structural studies. To this end, we developed a cross-linking strategy that involves mutation of two specific residues to Cys and thus allows formation of a disulfide bond between PS1 and the substrate. Using this approach, we obtained a cross-linked complex between a variant of human γ-secretase [with PS1-Q112C (Gln^{112}→Cys)] and APP-C83 (V695C). To avoid substrate cleavage, the catalytic residue Asp^{385} in PS1 was mutated to Ala. Because PS1 undergoes autoproteolysis during γ-secretase assembly to produce an N-terminal fragment (NTF) and a C-terminal fragment (CTF), these two fragments of PS1 in the γ-secretase were coexpressed. The final γ-secretase contains PS1 (NTF-Q112C, CTF-D385A), PEN-2, APH-1aL (a specific isoform of APH-1), and nicastrin. This γ-secretase was cross-linked to APP-C83 (V695C), and the complex was analyzed by cryo–electron microscopy (cryo-EM).

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RESULTS: The cryo-EM structure of the cross-linked human γ-secretase–APP-C83 complex was determined at an average resolution of 2.6 Å. The quality of the EM map allows unambiguous identification of the bound APP fragment, which traverses through the center of the γ-secretase TM domain. Compared to substrate-free γ-secretase, the flexible transmembrane helix 2 (TM2) of PS1 becomes ordered upon binding to APP-C83 and contributes to its recognition, and the C-terminal portion of TM6 of PS1 is unraveled into a rigid loop followed by a short α helix (designated as TM6a). The TM of APP closely interacts with five surrounding TMs (TM2, TM3, TM5, TM6, and TM7) of PS1. Notably, the APP sequences on the C-terminal side of the TM form a β strand, which, together with two APP-induced β strands of PS1, constitutes a hybrid β sheet on the intracellular side. This β sheet guides γ-secretase to the scissile peptide bond of APP just preceding the N terminus of the β strand. Mutations that compromise the hybrid β sheet result in abrogation of APP cleavage by γ-secretase. Notably, residues at the interface between PS1 and APP are heavily targeted by recurring AD mutations.

CONCLUSION: The structure of human γ-secretase bound to APP-C83 constitutes a framework for understanding the function and disease relevance of γ-secretase. This structure, together with that of γ-secretase bound to Notch, reveals contrasting features of substrate recognition, which may be applied toward the design of substrate-specific inhibitors. ■

The cryo-EM structure of human γ-secretase bound to APP at 2.6-Å resolution. (Left) Overall structure of the γ-secretase–APP-C83 complex. PS1, cyan; PEN-2, yellow; APH-1, pink; nicastrin (NCT), green; APP, blue. (Top right) Close-up view of the hybrid β sheet. The three-stranded β sheet comprises a β strand from APP and two β strands from the extended loop sequence between the NTF and CTF of PS1. (Bottom right) Close-up structural comparison between free APP (orange) and APP bound to γ-secretase (blue). The two initial cleavage sites of γ-secretase are marked by arrows.

The list of author affiliations is available in the full article online.
*These authors contributed equally to this work.
†Corresponding author. Email: shi-lab@tsinghua.edu.cn
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RESEARCH ARTICLE

STRUCTURAL BIOLOGY

Recognition of the amyloid precursor protein by human γ-secretase

Rui Zhou[1]*, Guanghui Yang[1]*, Xuefei Guo[1], Qiang Zhou[2,3], Jianlin Lei[1,4], Yigong Shi[1,2]†

Cleavage of amyloid precursor protein (APP) by the intramembrane protease γ-secretase is linked to Alzheimer's disease (AD). We report an atomic structure of human γ-secretase in complex with a transmembrane (TM) APP fragment at 2.6-angstrom resolution. The TM helix of APP closely interacts with five surrounding TMs of PS1 (the catalytic subunit of γ-secretase). A hybrid β sheet, which is formed by a β strand from APP and two β strands from PS1, guides γ-secretase to the scissile peptide bond of APP between its TM and β strand. Residues at the interface between PS1 and APP are heavily targeted by recurring mutations from AD patients. This structure, together with that of γ-secretase bound to Notch, reveal contrasting features of substrate binding, which may be applied toward the design of substrate-specific inhibitors.

The hallmark of Alzheimer's disease (AD) is the presence of amyloid plaques in the brain of AD patients (*1*, *2*). The primary components of the amyloid plaque are β-amyloid peptides (Aβs) derived from the amyloid precursor protein (APP) (*3*). The type I transmembrane (TM) protein APP is first cleaved by α- or β-secretase to generate a transmembrane fragment of 83 or 99 residues (APP-C83 or APP-C99), respectively (*4*, *5*) (fig. S1A). APP-C99 is then cleaved by γ-secretase through its endopeptidase activity to generate the 48-residue peptide Aβ48 or the 49-residue peptide Aβ49 (*6*–*8*). Subsequent cleavages of Aβ49 by the C-terminal peptidase activity of γ-secretase results in the sequential generation of Aβ46, Aβ43, and Aβ40 (*3*, *9*) (fig. S1A). Similarly, cleavages of Aβ48 lead to the production of Aβ45, Aβ42, and Aβ38. Of these, Aβ42 and Aβ43 are particularly prone to aggregation and formation of amyloid plaques (*3*, *10*, *11*). In addition to APP, the Notch receptor is also a substrate of α- and γ-secretases (*12*). After cleavage by α-secretase, the resulting transmembrane Notch fragment is cleaved by γ-secretase to generate an intracellular signaling domain (*13*). A model of stepwise substrate binding by γ-secretase was purposed on the basis of systematic photoaffinity mapping (*14*).

Human γ-secretase comprises four subunits: presenilin (PS), PEN-2, APH-1, and nicastrin (NCT) (*15*, *16*). As the catalytic subunit of γ-secretase, presenilin is an aspartyl protease with two catalytic Asp residues (*7*) and has two isoforms (PS1 and PS2). During γ-secretase assembly, PS1 undergoes autoproteolysis to produce an N-terminal fragment (NTF) and a C-terminal fragment (CTF) (*17*). PEN-2 is required for γ-secretase maturation, APH-1 stabilizes the complex (*18*), and NCT is thought to play a role in substrate binding (*19*). More than 200 AD-associated mutations have been identified in PS1, most of which result in elevated Aβ42/Aβ40 ratios (*20*).

The prevailing amyloid hypothesis postulates that the amyloid oligomers directly contribute to the development of AD (*11*, *21*–*23*), making inhibition of γ-secretase a potential therapeutic strategy for AD treatment (*24*–*26*). Unfortunately, perhaps because they also inhibit Notch cleavage (*27*), γ-secretase inhibitors cause severe side effects without any clear clinical benefits to AD patients. Here we report the cryo–electron microscopy (cryo-EM) structure of human γ-secretase in complex with a transmembrane APP fragment at 2.6-Å resolution. A hybrid β sheet between PS1 and the substrate is essential for the proteolytic activity of γ-secretase. Comparison of this structure with that of the γ-secretase–Notch complex (*28*) reveals distinctive features that may be exploited for development of substrate-specific inhibitors. Notably, the residues at the interface between PS1 and APP are heavily targeted for mutations in early-onset AD patients.

Preparation of a γ-secretase-APP complex

The γ-secretase–APP complex is transient and has long defied all efforts at isolation. We developed a chemical cross-linking strategy that aims to stabilize the transient γ-secretase–substrate complex. Using this approach, we obtained a cross-linked complex between γ-secretase (PS1-NTF-Q112C and CTF-D385A) and a 100-residue Notch fragment (Notch-100, P1728C) and determined its cryo-EM structure (*28*). The catalytic mutation D385A (Asp^{385}→Ala) in PS1 is required to prevent substrate cleavage by γ-secretase, and the NTF and CTF were coexpressed to mimic the outcome of PS1 autoproteolysis. On the basis of sequence alignment (fig. S1B), APP-C83 corresponds to Notch-100, with Val^{695} of APP corresponding to Pro^{1728} of Notch.

We applied the same strategy to generate four APP-C83 mutants, each with a cysteine substitution in a four-residue stretch, and individually examined their cross-linking efficiency with PS1 (NTF-Q112C, CTF-D385A) in γ-secretase (fig. S1C). Only APP-C83 (V695C) was completely cross-linked to PS1. Formation of a stable complex between γ-secretase (PS1-Q112C/D385A) and APP-C83 (V695C) strictly depended on cross-linking in the absence of the reducing agent dithiothreitol (DTT) (fig. S1D). Notably, the mutation V695C allowed retention of APP-C83 cleavage by γ-secretase (fig. S1E). This strategy allowed purification of a large amount of human γ-secretase (PS1-Q112C/D385A, PEN-2, APH-1aL, and NCT) cross-linked to its substrate APP-C83 (V695C) (Fig. 1A and fig. S1F). The disulfide bond in the purified complex could be reduced by DTT (Fig. 1A).

Structure of the γ-secretase–APP complex

We analyzed the cross-linked human γ-secretase–APP-C83 complex by single-particle cryo-EM and determined its structure at an average resolution of 2.6 Å (Fig. 1B, figs. S2 to S5, and table S1). In final atomic model, 34 residues from APP constitute two fragments: one spanning residues 688 to 693 and another spanning residues 699 to 726. An intervening five-residue stretch (residues 694 to 698) that includes the cross-linking site V695C is disordered in the EM density map (Fig. 1C and fig. S6, A and B). Unlike Notch, the APP TM fragment contains few bulky amino acids and no aromatic residues (fig. S1, A and B). Nonetheless, the side-chain assignment of the APP fragment spanning residues 699 to 726 was unambiguously assisted by four hydrophobic residues: Met^{706}, Ile^{718}, Met^{722}, and Leu^{723}. Similar to Notch (*28*), the structurally resolved APP sequence comprises an N-terminal loop, a TM helix, and a C-terminal β strand (Fig. 1C).

Compared with that of free APP (*29*), the TM helix in the γ-secretase–bound APP is unwound by one full helical turn at the C-terminal end (Fig. 1D). Consequently, three residues of the TM helix (Thr^{719}, Leu^{720}, and Val^{721}) in free APP adopt a fully extended conformation upon binding to γ-secretase. This structural change allows cleavage of the peptide bond to occur either between Thr^{719} and Leu^{720}, which results in Aβ48, or between Leu^{720} and Val^{721}, which yields Aβ49 (Fig. 1D). The extended conformation of the residues 718 to 721 is accompanied by a characteristic β strand (Val^{722} to Lys^{725}) that is present only in the γ-secretase–bound APP, not in free APP.

The APP TM helix, along with the β strand, traverse through a central pore formed by TM2, TM3, TM5, TM6, and TM7 of PS1 (Fig. 2A). TM2 of PS1, implicated in substrate recruitment

[1]Beijing Advanced Innovation Center for Structural Biology, Tsinghua-Peking Joint Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China. [2]Institute of Biology, Westlake Institute for Advanced Study, Westlake University, 18 Shilongshan Road, Xihu District, Hangzhou 310024, Zhejiang Province, China. [3]School of Life Sciences, Westlake University, 18 Shilongshan Road, Xihu District, Hangzhou 310024, Zhejiang Province, China. [4]Technology Center for Protein Sciences, Ministry of Education Key Laboratory of Protein Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China.
*These authors contributed equally to this work.
†Corresponding author. Email: shi-lab@tsinghua.edu.cn

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Fig. 1. Cryo-EM structure of human γ-secretase bound to a TM fragment of amyloid precursor protein (APP-C83). (**A**) The human γ-secretase–APP-C83 complex is stabilized by a disulfide bond between Cys^{112} of PS1-Q112C and Cys^{695} of APP-V695C. The purified γ-secretase–APP-C83 complex was visualized by SDS-PAGE through Coomassie staining (lower panel). The cross-linked fragment between PS1-NTF and APP-C83, which was formed in the absence of the reducing agent DTT, can be reduced by DTT in vitro, generating free PS1-NTF. For details, see fig. S1 and the Materials and methods section. MW, molecular weight. (**B**) Overall EM density map of human γ-secretase (gray) in complex with APP-C83 (blue). (**C**) Structure of APP-C83 from the γ-secretase–APP-C83 complex. The EM density for APP-C83 (left) and close-up views on four segments of APP-C83 (right) are shown. The contour level of the EM density for the entire APP-C83 is 5σ. The contour levels for the four focused regions are between 5.2σ and 6σ. (**D**) Structural comparison of the APP TM fragment in its free and γ-secretase-bound states. Compared to the free state (orange) [PDB ID: 2LLM (*29*)], the N and C termini of the γ-secretase–bound APP fragment (blue) undergoes marked changes. The N-terminal helix is replaced by a coil, and the C-terminal helix unwinds into an extended conformation to expose the potential cleavage sites and form a β strand on the intracellular side. Notably, cleavage after Thr^{719} or Leu^{720} results in Aβ48 or Aβ49, respectively. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

(*30*), is dynamic and disordered in substrate-free γ-secretase (*31*) but becomes ordered upon binding to the substrate and contributes to APP recognition. TM2 of PS1 and the TM of APP-C83 are located on the convex side of the horseshoe-shaped TM domain of γ-secretase (fig. S5, A and B). Most notably, the APP β strand forms an antiparallel three-stranded β sheet with two induced β strands from PS1: β1 (residues 287 to 290) at the C-terminal end of the structurally resolved portion of NTF and β2 (377 to 381) at the N-terminal end of CTF (Fig. 2A, inset, and fig. S5C). Formation of the hybrid β sheet is accompanied by a rearrangement of the C-terminal portion of TM6 in PS1: The TM6 helix in substrate-free γ-secretase is unraveled with substrate bound and after a rigid loop (residues 263 to 267) continues as a short α helix (designated as TM6a) (Fig. 2B). Notably, similar changes in TM6 are also observed in DAPT-bound γ- secretase (*32*) (fig. S6C), but formation of the hybrid β sheet is specific to substrate-bound γ-secretase.

These structural changes are stabilized by intramolecular interactions (Fig. 2C). In particular, Arg^{278} and Glu^{280}, which are located in the loop connecting TM6a and the strand β1, orchestrate a network of hydrogen bonds (H-bonds). The carboxylate side chain of Glu^{280} makes a bifurcated H-bond to the hydroxyl groups of Tyr^{154} and Tyr^{159}, both from TM2 of PS1. These interactions are buttressed by two additional H-bonds from Arg^{278} to Glu^{280} and Tyr^{159} (Fig. 2C). Consistent with the importance of these interactions, the mutation E280A has been observed in hundreds of early-onset AD patients (*33*, *34*). In addition, Leu^{271} and Thr^{274} from TM6a make van der Waals contacts to Val^{151} and Tyr^{154}. Consistent with the structural observations, Tyr^{154}, Val^{271}, and Thr^{274} are all targets of AD-associated

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Fig. 2. Structural changes of PS1 and APP-C83 upon association. (**A**) Overall structure of PS1 bound to APP-C83. The TM of APP-C83 is surrounded by TM2, TM3, TM5, TM6, and TM7 of PS1. In contrast to the substrate-free state (*31*), TM2 of PS1 can be clearly identified in the substrate-bound state. A hybrid β sheet is formed on the intracellular side, between the β strand from APP and two β strands (β1 and β2) from loop 2 of PS1. The catalytic residues are shown in red. The EM density of the β sheet is shown at a contour level of 6.5σ. (**B**) Structural comparison of TM6 between the APP-bound state (cyan) and substrate-free state [gray; PDB ID: 5A63 (*31*)]. Superimposition of the two structures reveals pronounced translocation of the C-terminal portion of TM6 toward the bound substrate APP-C83. (**C**) Close-up view of the newly formed helix TM6a. H-bonds are represented by red dashed lines. TM6a interacts with TM2 through a combination of H-bonds and van der Waals contacts. Glu^{280} appears to anchor this interface by contributing three H-bonds.

mutations, which result in abrogation of APP-C99 cleavage in vitro by the corresponding γ- secretase variants (*20*).

APP recognition by human γ-secretase

The N-terminal half of the APP TM helix is partially exposed to lipid membrane and thus makes sparse interactions with surrounding residues in PS1 (Fig. 3A). Met^{706} and Val^{707} of APP make van der Waals contacts to Tyr^{240} and Ile^{114} of PS1, respectively; whereas Thr^{714} and Val^{715} of APP closely stack against the hydrophobic residues Met^{146} and Trp^{165}. These interactions appear to be brought into registry by a specific H-bond between the carbonyl oxygen of Ile^{712} from APP and the hydroxyl group of Ser^{169} from TM3 of PS1 (Fig. 3A). The hydroxyl group of Ser^{169} is also within H-bond distance of the carbonyl oxygen of Trp^{165}.

Compared to the N-terminal half, the C-terminal half of the APP TM helix mediates relatively dense van der Waals contacts (Fig. 3B). Val^{717} of APP is nestled in a shallow hydrophobic pocket formed by three PS1 residues: Phe^{237}, Ile^{387}, and Phe^{388}. Ile^{718} of APP closely contacts Met^{146}, Thr^{147}, and Leu^{268} of PS1, whereas Leu^{720} interacts with Ala^{434}, Leu^{435}, and Gly^{384}. Notably, Ala^{434} and Leu^{435} are part of the PAL motif, which has previously been implicated in substrate recognition (*35*). These interactions are anchored by a H-bond between the carbonyl oxygen of Thr^{719} from APP and the amide group of Gly^{384} from PS1 (Fig. 3B). The mutation G384A in PS1 causes early-onset AD (*36*); compared with wild-type enzyme, the γ-secretase variant that contains the mutation G384A in PS1 substantially increases the Aβ42/Aβ40 ratio (*20*).

The β strand of APP interacts with strand β2 and the PAL motif of PS1, mostly through main-chain H-bonds (Fig. 3C). These interactions, together with those involving the C-terminal half of the APP TM helix, facilitate the extended conformation of the APP residues 718 to 721. The carboxylate of the catalytic residue Asp^{257} is positioned approximately 6 to 7 Å away from the scissile peptide bond between residues 719 and 720 or 720 and 721 (Fig. 3D). In our study, the other catalytic residue (Asp^{385}) in PS1 was mutated to Ala to prevent cleavage of the bound APP substrate. It is possible that such a mutation might also lead to slight perturbation of the local conformation in the active site.

The TM segment of APP is accommodated in a cut-through channel of PS1 (fig. S7A). The cleavage products of APP-C99 by γ-secretase follow two lines: Aβ49-Aβ46-Aβ43-Aβ40 and Aβ48-Aβ45-Aβ42-Aβ38. For either line, the C-terminal residues are located on the same side of the TM helix (fig. S7B). Analysis of the binding pockets for the side chains of these residues reveals intriguing features (fig. S7, C to F). For example, the mutation I716F in APP is known to markedly elevate the Aβ42/Aβ40 ratio (*37*). In the structure, the binding pocket of Ile^{716} is large enough to accommodate the aromatic side chain of Phe (fig. S7E); the mutation I716F may favor production of Aβ48 through stabilization of the corresponding APP conformation. In addition, the binding pocket for Leu^{720}-Val^{721}-Met^{722} of APP, which is located next to the γ-secretase cleavage site, appears to follow the large-small-large pattern as previously described (*38*) (fig. S7, G and H).

To corroborate the structural observations, we generated four γ-secretase mutants, each with a deletion or mutation in PS1, and examined their activity toward the APP-C83 substrate (Fig. 3E). Deletion of β1 (residues 288 to 290), β2 (residues 377 to 381), or the PAL motif (residues 433 to 435) crippled the proteolytic cleavage of APP-C83. The missense mutation L432P, which presumably affects the local conformation and stability of the APP β strand, also abolished the activity.

Differential recognition of APP and Notch

Structural elucidation of APP recognition by human γ-secretase allows comparison with Notch recognition (*28*). Although the global conformation of γ-secretase remains unchanged between the APP- and Notch-bound states, substantial structural rearrangements are observed in the substrate-binding regions of PS1 and are likely induced by differential substrate binding (fig. S8A). The C-terminal half of TM2 and the N-terminal half of TM3, along with the short intervening linker sequence, undergo noticeable shift. In addition, the loop sequences preceding TM2 also exhibit different conformations between the APP- and Notch-bound states.

These differences are caused by the distinct sequence features of APP and Notch. Unlike the Notch TM helix that contains four Phe residues and four β-branched residues, the APP helix contains no aromatic residues and 11 β-branched residues (fig. S1, A and B). Consequently, compared with Notch, the APP helix exhibits distinctive surface features and appears slightly smaller. Two distinct sets of amino acids from

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Fig. 3. Recognition of APP-C83 by human γ-secretase. (**A**) Close-up view of the interactions of the N-terminal half of the TM helix of APP-C83 (blue) with surrounding structural elements from PS1 (cyan). H-bonds are represented by red dashed lines. A conserved H-bond between the side chain of Ser[169] and the carbonyl oxygen of Ile[712] appears to anchor this interface. (**B**) Close-up view of the interactions of the C-terminal half of the TM helix and the ensuing residues of APP-C83 (blue) with surrounding structural elements from PS1 (cyan). This interface is anchored by a conserved H-bond between the amide group of Gly[384] of PS1 and the carbonyl oxygen of Thr[719] of APP-C83. (**C**) Close-up view of the interactions surrounding the APP β strand. The PAL motif (Pro[433]-Ala[434]-Leu[435]) from PS1, which is implicated in substrate binding, stabilizes the APP β strand and orients the scissile peptide bonds. (**D**) Close-up view of the active site of PS1 and the cleavage site in APP-C83. The active site residues in PS1, Asp[257] and Ala[385] (replacing Asp[385]), are displayed in ball-and-stick representation. Cleavage of Thr[719]-Leu[720] or Leu[720]-Val[721] results in Aβ48 or Aβ49, respectively. (**E**) Formation of the β sheet is indispensable for γ-secretase cleavage. Deletion of β1 (residues 288 to 290), β2 (residues 377 to 381), or the PAL motif (residues 432 to 434) in PS1 leads to abrogation of the cleavage activity of γ-secretase. AICD, APP intracellular domain; WT, wild type.

PS1 are employed to interact with the TM helix from APP and Notch. Leu[85], Thr[147], and Ile[287] contribute to APP but not Notch binding (fig. S8B), whereas Phe[176] and Phe[177], along with eight other PS1 residues, directly interact with residues from Notch but not APP (fig. S8C). A set of residues, exemplified by Ser[169] and Gly[384], is involved in binding to both APP and Notch (fig. S8D). The TM helix from both APP and Notch appears to be anchored by a pair of conserved H-bonds donated by the hydroxyl group of Ser[169] and the amide group of Gly[384] (fig. S8E). The acceptors of these H-bonds are carbonyl oxygen atoms.

Differences in substrate recognition might be exploited to facilitate the discovery of substrate-specific inhibitors of γ-secretase. For both substrates, the N-terminal half of the TM helix is exposed to lipid membrane (Fig. 4A), with five consecutive bulky residues (LHFMY) in Notch (fig. S1A). Because these bulky residues in Notch occupy more space than the corresponding residues in APP, an inhibitor could target this region of PS1 to selectively block Notch but not APP binding. The C-terminal half of the TM helix shows alternating size differentials between APP and Notch. Along the helical axis, the Val[715]-Ile[716] segment of APP is smaller than the Phe[1748]-Phe[1749] segment in Notch (Fig. 4B). In contrast, the Ile[718]-Thr[719]-Leu[720] segment of APP is considerably bulkier than the Gly[1751]-Cys[1752]-Gly[1753] segment of Notch (Fig. 4C). It is conceivable that a small inhibitor bound to this region of PS1 may selectively inhibit the cleavage of APP but not Notch. Notably, this latter segment encompasses the scissile peptide bond that generates Aβ48. Systematic comparison of the surface features between the APP and Notch TM fragments reveals candidate binding sites for such an inhibitor (Fig. 4D).

AD-associated mutations at the PS1-APP interface

Structure determination of the human γ-secretase–APP complex allows assessment of the impact of AD-associated mutations on the interactions between γ-secretase and APP. On one hand, 247 AD-associated mutations in PS1 have been reported to affect 136 amino acids (www.alzforum.org/mutations/). Of these 136 residues, 59 are targeted for mutations to two or more types of amino acids (recurring mutations). One hundred twenty-five of the 136 affected residues, or 92% of the total, and the vast majority of the 59 mutational hotspot residues can be mapped onto the structurally resolved regions of PS1 (Fig. 5A). On the other hand, 30 AD-associated mutations in APP have been identified to affect 17 residues, of which 11 (65% of the total) can be seen in our structure. Among the 11 identified residues, two reside in the extracellular loop and nine are located in the TM helix and β strand (Fig. 5, B and C).

The 59 mutational hotspot PS1 residues can be classified into four distinct groups. The first group of six residues is located in the extended sequences on the extracellular side preceding TM2 (loop-1) (Fig. 5A). These residues likely play a role in substrate recruitment and delivery into

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Fig. 4. Differential recognition of Notch and APP by human γ-secretase. (**A**) Overall comparison of APP (marine) and Notch (orange) bound to PS1 (gray). PS1 is shown in surface representation. (**B**) Close-up view of the binding site of PS1 for the Val[715]-Ile[716] segment of APP or the Phe[1748]-Phe[1749] segment of Notch. The APP segment is considerably smaller than that of Notch. (**C**) Close-up view of the binding site of PS1 for the Ile[718]-Thr[719]-Leu[720] segment of APP or the Gly[1751]-Cys[1752]-Gly[1753] segment of Notch. The APP segment is considerably larger than that of Notch. (**D**) Systematic analysis of the side-chain features of the APP-TM and Notch-TM fragments. Shown at left are two views of the superimposition of the APP-TM (blue) and Notch-TM (orange) fragments in their surface representation. The four boxed segments are depicted in the middle and right panels. Bulky residues are labeled.

the active site (Fig. 5D). The second group of 19 residues has side chains pointing into the substrate-binding pore (Fig. 5D). The third group of 17 residues is located in the region that undergoes marked conformational changes upon substrate binding (i.e., TM6a, TM2, and the N-terminal portion of TM3) (Fig. 5E). Mutations in the second and third groups are likely to alter interactions with the substrate and/or hinder conformational changes induced by substrate binding. The fourth group—the remainder of the 59 hotspot residues—does not belong to any of the above three groups. But mutation of any of these residues, exemplified by Gly[206] and Gly[209], may affect residues of the other three groups. For example, mutation of Gly[206] or Gly[209] likely affects the conformation of the adjacent residue Leu[174], which may propagate to Ser[169] or Phe[177], both in the second group (Fig. 5F).

Discussion

The structure reported here reflects that of a mutated γ-secretase cross-linked to a mutated substrate. As such, the structure may represent a snapshot, and other structural variations of the γ-secretase–substrate complex are possible. Nevertheless, the atomic structure of the human γ-secretase–APP-C83 complex provides a physical basis for understanding the consequences of AD-associated mutations. The APP residues targeted for mutation in AD patients are clustered in the C-terminal half of the APP TM helix and the β strand, which are sequentially positioned before and after the scissile peptide bonds, respectively (Fig. 5A). Notably, the vast majority of the mutational hotspot PS1 residues appear to directly or indirectly affect APP binding and cleavage. In particular, a sizable fraction of these residues are clustered in the regions surrounding the C-terminal half of the APP TM helix and the β strand (Fig. 5, B and C). This analysis implicates a role of APP recruitment and cleavage in the pathogenesis of AD.

Despite the differences in substrate recognition, design of substrate-specific inhibitors of γ-secretase is challenging for two reasons. First, APP and Notch, each comprising a TM helix and a β strand, bind to the same general location in γ-secretase. Second, the recognition mechanism between APP and Notch shares two common themes. In both cases, the TM helix is anchored

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Fig. 5. PS1 residues involved in the recruitment and cleavage of APP are predominantly targeted for recurring mutations in AD patients. (**A**) Mapping of AD-associated mutations onto the structure of human γ-secretase bound to APP-C83. AD-associated mutations in APP are shown in orange. AD-associated recurring mutations in PS1 are highlighted in cyan (*20*). Most recurring mutations are located along the binding pore of APP-TM. (**B**) Close-up view of the interface between the C-terminal half of the APP TM helix and surrounding PS1 elements. All residues shown in the stick model are targeted for recurring mutations in AD patients. (**C**) Close-up view of the interface between the β strand of APP and the surrounding elements of PS1. All residues shown in the stick model are targeted for recurring mutations in AD patients. (**D**) 19 residues in PS1 that are targeted for recurring mutations in AD patients place their side chains toward the interior of the substrate-binding pore. (**E**) A close-up view on the residues that appear to stabilize the structural arrangement upon APP binding. These residues are targeted for recurring mutations in AD patients. (**F**) A close-up view on the PS1 residues Leu^{174}, Gly^{206}, and Gly^{209}. These three residues are subject to recurring mutations in AD patients. Each mutation likely affects the local conformation, which propagates to those residues that directly contribute to substrate binding.

through a pair of conserved H-bonds, and substrate cleavage is oriented through formation of a hybrid β sheet between the substrate and PS1. Nonetheless, careful analysis reveals small but important structural differences between APP and Notch, especially in the regions toward the C-terminal end of the TM helix (Fig. 4). An inhibitor that selectively targets APP cleavage is likely to be bimodal. One end of the inhibitor should ideally bind to the surface specific to APP-bound γ-secretase, which, for example, may involve the N-terminal portion of TM3. The other end of the inhibitor, presumably small in size, may be fitted into the space that would be occupied only by the bulkier residues of APP, which, for instance, could be the region surrounding Ile^{718}-Thr^{719}-Leu^{720}.

APP-C99, but not APP-C83, is the precursor to the aggregating β-amyloid peptides. In this study, APP-C83, rather than APP-C99, was chosen to be the substrate of γ-secretase. This choice of substrate is based on sequence alignment between APP-C99 and Notch-100 as well as the fact that γ-secretase interacts with APP-C83 more strongly than with APP-C99 (*39*). Consequently, the endopeptidase cleavage of APP-C99 by γ-secretase is less efficient compared with that of APP-C83 (fig. S9, A and B). However, the observed interactions between APP-C83 and PS1 are likely be identical to those in the γ-secretase–APP-C99 complex. Compared to APP-C83, the extra 16 amino acids at the N-terminus of APP-C99 may play an additional regulatory role in formation of the γ-secretase–substrate complex and possibly catalysis (*14*, *40*).

Compared to free γ-secretase, the overall structures of the other three subunits (PEN-2, APH-1, and NCT) of APP-bound γ-secretase remain largely unchanged (fig. S6, D to H). The putative substrate-binding residue Glu^{333} and its surrounding structural elements also show few changes between the substrate-free and APP-bound states of γ-secretase (fig. S6E). In our structure, the N-terminal two residues Leu^{688} and Val^{689} of APP-C83 directly interact with surrounding residues from NCT (fig. S9C). It is possible that residues on the N-terminal side to Lys^{687} may modulate this interaction. Consistent with this analysis, in the absence of DTT, γ- secretase forms a stable complex with APP-C83 but not with APP-C99 (fig. S9D).

The structure of human γ-secretase bound to the amyloid precursor protein lends strong support to the helix-unwinding model of successive substrate cleavage by γ-secretase (*20*). More importantly, this structure allows comparison of APP and Notch recognition by γ-secretase and rationalization of AD-associated mutations. As such, this structure serves as an important framework for discovery of substrate-specific inhibitors of γ-secretase and for understanding the biological functions of γ-secretase as well as the disease mechanisms of AD.

Materials and methods

Clones and plasmids

The coding DNA sequences for human PS1 and its variants, APH-1aL, PEN-2, NCT, and the APP fragments were individually cloned into the pMLink vector as previously described (*41*). All plasmids used for transfection of mammalian cell were prepared using the EndoFree Plasmid Maxi Kit (Cwbiotech).

Rationale and design of a γ-secretase–APP complex

To obtain a stable γ-secretase–C83 complex, we sought to stabilize the enzyme-substrate complex through an engineered disulfide bond as reported in the case of the γ-secretase–Notch complex (*28*). The APP sequences comprise a large

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extracellular domain, a TM region, and a short intracellular domain known as AICD (fig. S1A). On the basis of sequence alignment, we chose a four-residue stretch of APP-C83 that corresponds to the region of exhaustive Cys mutation in Notch (fig. S1B). Because PS1 undergoes autoproteolysis during γ-secretase assembly to produce an NTF and a CTF, the NTF and CTF of PS1 were coexpressed together with the other three subunits (PEN-2, APH-1aL, and NCT) to generate recombinant γ-secretase. The catalytic residue Asp^{385} was mutated to Ala in PS1 to avoid substrate cleavage. Then we generated four APP-C83 mutants, each with a cysteine substitution in the four-residue stretch, and individually examined their cross-linking efficiency with PS1 (NTF-Q112C, CTF-D385A) in γ-secretase (fig. S1C). Only APP-C83 (V695C) was completely cross-linked to PS1. Formation of a stable complex between γ-secretase (PS1-Q112C/D385A) and APP-C83 (V695C) strictly depended on cross-linking in the absence of the reducing agent DTT (fig. S1D). Importantly, the mutation V695C allowed retention of APP-C83 cleavage by the wild-type γ-secretase (fig. S1E). Nonetheless, the cleavage activity is reduced compared to the wild-type APP-C83. This strategy allowed purification of a large amount of human γ-secretase (PS1-Q112C/D385A, PEN-2, APH-1aL, and NCT) cross-linked to its substrate APP-C83 (V695C) (Fig. 1A and fig. S1F).

Protein expression and purification

Human γ-secretase (PS1-NTF-Q112C, PS1-CTF-D385A, PEN-2, APH-1aL, NCT) and APP-C83 (residues 688-771, V695C) were coexpressed in HEK293 cells and purified as previously described (28). Cell membranes were resuspended in the lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl) supplemented with 0.1% (w/v) digitonin and 1% (w/v) CHAPSO. PEN-2 has an N-terminal FLAG tag for affinity purification. APP-C83 is tagged with Myc and 6xHis at its C terminus. The γ-secretase–APP-C83 complex eluted from the affinity column was further purified by gel filtration (Superose-6, GE Healthcare) in the lysis buffer supplemented with 0.1% digitonin.

For each of the γ-secretase variants used in the proteolytic activity assay, PS1 carries a specific missense mutation or deletion. Purification of such γ-secretase variants was performed as previously described (31). PS1 was detected by an anti-PS1 monoclonal antibody (Merck), and the Myc tag was detected by an anti-Myc monoclonal antibody (Cwbiotech, Beijing).

Electron microscopy

Cryo-EM samples were prepared as described (31). 4-μl aliquots of recombinant human γ-secretase cross-linked to APP-C83 were applied to glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3, 300 mesh). The grids were blotted for 3 s and flash frozen in liquid ethane using Vitrobot Mark IV (FEI). The sample was imaged on an FEI Titan Krios transmission electron microscope equipped with a Cs corrector, operating at 300 kV with a nominal magnification of 105,000×. Images were recorded by a Gatan K2 Summit direct electron detector and a Gatan GIF Quantum energy filter (slit width: 20 eV) using the super-resolution mode. Defocus values varied from −1.0 to −2.2 μm. Each image was dose fractionated to 32 frames with a total electron dose of ~50 $e^-/Å^2$ and a total exposure time of 5.6 s. AutoEMation II (developed by Jianlin Lei) (42) was used for automated data collection. All stacks were motion corrected using MotionCor2 (43) with the binning factor of 2, resulting in a pixel size of 1.091 Å. The defocus values were estimated using Gctf (44) and dose weighing was performed concurrently (45).

Cryo-EM image processing

In total, 6838 movie stacks were recorded (fig. S2). After motion correction and CTF estimation, 6360 micrographs were selected. 3,575,237 particles were autopicked from these 6360 movie stacks using RELION-2.0 (46–49). After two-dimensional (2D) classification, 2,925,279 particles were selected and subjected to 3D classification. The γ-secretase EM map (EMD-3061) was low-pass filtered to 20 Å to generate an initial model (31). The selected particles were subjected to 25 iterations of global angular search 3D classification. Each of the 25 iterations has one class and a step size of 7.5°. For the last iteration (iteration 25) of the global search, the local angular search 3D classification was executed with a class number of five, a step size of 3.75°, and a local search range of 15°. The resulted classes of local search were used to generate multireferences.

For the last six iterations (iterations 20 to 25) of the global search, multireference 3D classification was performed, each with a class number of five, a step size of 3.75°, and a local search range of 15° for 10 iterations. For the last five iterations of the multireference classification, particles from the "good" classes (i.e., those with additional EM density in PS1) were merged and duplicated particles were removed. After the multireference 3D classification, 1,076,837 particles (36.8% of all selected particles after 2D classification) were subjected to another round of multireference 3D classification. Particles from good classes were applied to autorefinement, resulting in a 3.0-Å map. The box size was then changed from 200 pixels to 320 pixels, after which 3D autorefinement improved the resolution to 2.6 Å on the basis of the Fourier shell correlation (FSC) 0.143 criterion (50). The FSC curves were corrected for the effects of a soft mask using high-resolution noise substitution (51). Local resolution variations were estimated using RELION-2.0 (46).

Model building and structure refinement

The initial model used for the γ-secretase–APP-C83 complex was the substrate-free γ-secretase (31). The structure was first refined in real space using PHENIX with secondary structure and geometry restraints (52). APP-C83 was built de novo from a poly-Ala model. The atomic model was manually improved using COOT (53). Sequence assignment was guided by relatively bulky residues such as Met and Ile. Sixteen glycosylation sites were identified on the basis of clear features in the EM density map. Three cholesterols and two phosphatidylcholines were found to surround the TM domain of γ-secretase. Several EM density lobes resemble phospholipids, but the quality of these densities was insufficient for their assignment. The final atomic model was refined in real space using PHENIX (54). The final atomic model was evaluated using MolProbity (55).

γ-secretase proteolytic activity assay

APP-C83 or APP-C99 with a C-terminal Myc-His_6 tag was overexpressed in *Escherichia coli* and purified using a Ni^{2+}-NTA column. The eluted materials were then applied to gel filtration (Superdex-200, GE Healthcare) in the lysis buffer supplemented with 0.5% (w/v) CHAPSO. Purified substrate was mixed with purified γ-secretase in the lysis buffer supplemented with 0.2% (w/v) CHAPSO, 0.1% (w/v) phosphatidylcholine, 0.025% (w/v) phosphatidylethanolamine, and 0.00625% (w/v) cholesterol. The reaction was allowed to proceed at 37°C for 4 hours. The concentrations of all γ-secretase variants were determined using the Bradford method and confirmed by applying aliquots of the variants to SDS–polyacrylamide gel electrophoresis (PAGE). The final γ-secretase concentration was 60 nM in the cleavage assays. The final substrate concentration in the cleavage assay is ~8 μM. The cleavage product AICD-Myc was detected by an anti-Myc monoclonal antibody (Cwbiotech, Beijing).

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S9
Table S1

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