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Unraveling the Complexity of γ-Secretase

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Abstract

γ-Secretase was initially defined as a proteolytic activity that cleaves within the transmembrane of the amyloid precursor protein (APP) to produce the amyloid β-peptide of Alzheimer's disease. The discovery of mutations in APP and the presenilins associated with familial Alzheimer's disease and their effects on APP processing dovetailed with pharmacological studies on γ-secretase, leading to the revelation that presenilins are unprecedented membrane-embedded aspartyl proteases. Other members of what became known as the γ-secretase complex were subsequently identified. In parallel with these advances, connections between presenilins and Notch receptors essential to metazoan development became evident, resulting in the concurrent realization that γ-secretase also carries out intramembrane proteolysis of Notch as part of its signaling mechanism. Substantial progress has been made toward elucidating how γ-secretase carries out complex processing of transmembrane domains, how it goes awry in familial Alzheimer's disease, the scope of its substrates, and the atomic details of its structure. Critical questions remain for future study, toward further unraveling the complexity of this unique membrane-embedded proteolytic machine and its roles in biology and disease.

Keywords

protease; membrane proteins; amyloid; Notch; Alzheimer's disease

1. Introduction

γ-Secretase is a proteolytic ensemble of four integral membrane proteins that carries out hydrolysis within the lipid bilayer, processing the transmembrane domain (TMD) of over 90 known substrates [1–3]. With so many substrates, γ-secretase is considered "the proteasome of the membrane" [4], clearing out membrane protein stubs remaining after ectodomain shedding. In certain cases, these TMD cleavage events are part of cell signaling pathways, including Notch signaling essential to development of all metazoans [5]. In one particular case, involving the amyloid precursor protein (APP), complex TMD cleavage to the amyloid

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β-peptide (Aβ) by γ-secretase is aberrant in dominantly inherited, early-onset familial Alzheimer's disease (FAD) [6].

Thus, the complexity of γ-secretase can be appreciated on many levels. One level is structural and biochemical. The four membrane components of the enzyme assemble together to become an active protease capable of bringing water to the active site located within the hydrophobic environment of the lipid bilayer [7–9]. The protease complex must find, recognize, and unwind the α-helical TMD substrates to expose the scissile amide bond for hydrolysis. γ-Secretase then carries out processive proteolysis to trim down initially formed membrane-bound products to shorter secreted forms [10–14]. All these biochemical events are incompletely understood in mechanistic and structural detail.

A second level of complexity is the myriad and essential roles of γ-secretase in biology. Knockout of enzyme components results in embryonic lethal phenotypes that closely resemble those seen upon knockout of Notch receptors [15, 16], suggesting that the role of γ-secretase in Notch signaling is its most important biological function and likely the primary driver of evolution of the protease complex. Nevertheless, other key signaling pathways involve γ-secretase processing of TMD substrates [17–19].

A third level of complexity is the role of γ-secretase in human disease, particularly FAD. More than 20 years after the discovery of FAD missense mutations in presenilin [20, 21], the catalytic component of γ-secretase [22], the question of how these mutations alter γ-secretase activity to trigger FAD is still controversial. The issue is further inflamed by the many failures of candidate Alzheimer therapeutics in clinical trials, with no new drug approvals in the U.S. since 2003. As many of these clinical candidates have targeted Aβ, skepticism of the "amyloid hypothesis" of Alzheimer pathogenesis has grown [23].

This overview for the special issue on γ-secretase will recount the critical findings that ultimately led to the discovery of presenilin as the catalytic component of the protease, the identification of other members of the complex, and the realization of the connection between γ-secretase and Notch signaling. Advances in understanding the roles of γ-secretase in biology and disease, particularly FAD, will also be discussed, along with major progress in elucidating the atomic structure of the γ-secretase complex and how the enzyme recognizes TMD substrates.

2. Early Groundwork

γ-Secretase was initially defined as a proteolytic activity that cleaves within the APP TMD, after ectodomain shedding by α- or β-secretase, to produce either the 3-kDa p3 or the 4-kDa Aβ, respectively (Fig. 1) [24]. The Aβ peptide is amphipathic, highly aggregation prone, and found deposited in the brain in Alzheimer's disease. That the proteolysis takes place in the TMD and the proteolytic product was observed pathologically led to the suggestion that this cleavage was aberrant, occurring after some disruption that pulls the APP fragments out of the membrane. The discovery that Aβ is secreted naturally from cultured cells changed this notion [25, 26], although the idea of proteolysis within the membrane still seemed implausible.

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The discovery in 1991 of missense mutations in APP associated with FAD pointed to alterations in normal Aβ production as pathogenic [27, 28]. These mutations were found in and around the small Aβ region of APP, and they had the effect of either (1) increasing total Aβ production by increasing cleavage by β-secretase, (2) increasing the aggregation tendency of Aβ, or (3) shifting the preferred site of APP TMD cleavage by γ-secretase to increase the proportion of highly aggregation-prone 42-residue Aβ (Aβ42) over the more soluble 40-residue peptide (Aβ40) [29].

In 1995, the first missense mutations in genes encoding presenilin-1 and −2 (PSEN1, PSEN2) associated with FAD were discovered, providing the first important clue to the identity of the mysterious γ-secretase [20, 21]. The presenilins are multi-pass membrane proteins, of completely unknown function at that time. The only known homolog was an obscure gene in *Caenorhabditis elegans* called *spe-4* involved in spermatogenesis [30]. The connection to Alzheimer's disease was entirely unclear. However, the FAD presenilin mutations were soon found to increase the ratio of Aβ42 to Aβ40 [31–36], suggesting that pathogenesis is due to altering γ-secretase cleavage of APP substrate to increase the proportion of the aggregation-prone Aβ.

Presenilins were also quickly connected to signaling from the Notch family of cell-surface receptors. Through genetic screening, the *C. elegans* gene *sel-12*, related to *spe-4* and human *PSEN1* and *PSEN2*, was found to facilitate signaling from lin-12 and glp-1, worm Notch receptor orthologs [37]. Moreover, knockout of *PSEN1* in mice resulted in an embryonic lethal phenotype closely resembling that seen upon knockout of *Notch1* [15, 16]. Thus, presenilins were closely linked with a developmental signaling pathway critical to cell differentiation. Notch1 protein was then found to be cleaved within its single TMD to release an intracellular domain (NICD; Fig. 1) that translocates to the nucleus and activates transcription factors critical to development in metazoans [38]. NICD release and translocation was soon after found to be presenilin-dependent [39, 40].

Further clues to the biochemical function of presenilin were provided by the findings that presenilin itself undergoes endoproteolytic processing into an N-terminal fragment (NTF) and C-terminal fragment (CTF), that the formation of these fragments is tightly regulated by limiting cellular factors, and that NTF and CTF are stable, remain associated, and assemble into a high-molecular-weight complex [41–48]. These findings together suggested that the presenilin holoprotein is a precursor and that the functional form of presenilin is the associated NTF and CTF. Subsequently, the culturing of embryonic cells from *PSEN1* knockout mice revealed that APP TMD processing by γ-secretase to Aβ was dramatically deficient [49], with remaining Aβ production attributed to PSEN2 (later shown to be correct) [50, 51]. Thus, presenilin was found to be essential for γ-secretase activity. However, because it bore no resemblance to any known protease, presenilin was suggested as likely a regulator of γ-secretase activity [49], analogous to an activating factor involved in transmembrane processing of the sterol regulatory element binding protein [52].

In parallel with the discovery of the apparent requirement of presenilin in the cleavage of APP by γ-secretase were pharmacological studies aimed at characterizing this unidentified protease. Peptidomimetics based on the APP TMD were developed, containing a protease

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inhibitory motif, a difluoroketone [53]. This motif was installed in place of the amide bond that is cleaved to produce Aβ42 in short peptidomimetics, thereby creating the first reported substrate-based inhibitor of γ-secretase. While providing a useful chemical tool for the study of γ-secretase and its biological roles, this compound was not informative about the proteolytic mechanism. However, follow-up development of related difluoroalcohol peptidomimetics [54] provided the first clear evidence that γ-secretase is an aspartyl protease: the difluoroalcohol motif is only known to inhibit aspartyl proteases, not other mechanistic classes of proteases. A subsequent report of a hydroxyethyl-type peptidomimetic, a related type of transition-state analog, as an inhibitor of γ-secretase offered further evidence for this hypothesis [55].

3. Intramembrane Proteolysis by Presenilin

The evidence that γ-secretase is an aspartyl protease along with the finding that PSEN1 is critical for γ-secretase activity raised the question of whether presenilin might be an aspartyl protease responsible for the cleavage of the APP TMD [22]. Inspection of the sequences of the known presenilins led to the identification of two completely conserved aspartates predicted to reside in TMD 6 and TMD7 (Fig. 2). Mutation of either aspartate in PSEN1 resulted in diminished Aβ production and increased levels of APP CTF γ-secretase substrates upon stable expression in mammalian cells as well as in microsomes isolated from these cells. Even conservative mutation to glutamate led to inhibition of γ-secretase processing of APP CTF substrates.

Unexpectedly, mutation of either TMD aspartate also prevented normal endoproteolysis of PSEN1 into NTF and CTF. To test if the aspartates are critical for γ-secretase activity even without the need for PSEN1 NTF and CTF formation, these aspartates were mutated in the E9 variant of PSEN1 [22]. This variant, associated with FAD, lacks exon 9, which encodes the endoproteolytic site within the large loop of PSEN1 between TMD 6 and TMD7. E9 PSEN1 is not cleaved into NTF and CTF [41], yet still assembles into stable high-molecular-weight complexes [47] and acted as a functional presenilin (i.e., could substantially rescue an egg-laying deficiency in *C. elegans* caused by *sel-12* mutation) [56]. Mutation of one of the TMD aspartates in E9 PSEN1 likewise blocked γ-secretase cleavage of APP upon expression in mammalian cells, demonstrating the separate requirement of the aspartates for PSEN1 endoproteolysis and γ-secretase activity [22].

Taken together, these findings suggested that presenilin is a novel membrane-embedded aspartyl protease that cleaves the APP TMD to produce Aβ peptides and further suggested that full-length presenilin undergoes autoproteolysis into NTF and CTF. PSEN1 NTF and CTF each contribute one of the conserved TMD aspartates essential for proteolysis, suggesting that the active site of γ-secretase resides at the interface between these two subunits. Overexpression of wild-type PSEN1 did not lead to increased Aβ production, suggesting that the limiting cellular factors gating the formation of stable presenilin NTF/CTF heterodimers might also control the activation of γ-secretase.

This mutagenesis study was quickly confirmed in both PSEN1 and PSEN2 [57, 58]. Skepticism for the presenilin-as-protease hypothesis remained; however, this was soon

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largely allayed by identifying the interface between presenilin NTF and CTF as the binding site for aspartyl-protease transition-state analog inhibitors of γ-secretase [59, 60]. Such inhibitors were converted to biotinylated affinity reagents designed to covalently crosslink to their protein target containing the active site of γ-secretase. Detection of the biotinylated protein targets revealed presenilin NTF and CTF, suggesting that the active site of γ-secretase resides between these two subunits, consistent with the contribution by each subunit of one of the critical conserved TMD aspartates.

Concurrent with the inhibitor affinity labeling experiments was the discovery of another polytopic membrane protein serving as an aspartyl protease. The bacterial type 4 prepilin peptidases (TFPPs) were found to contain two conserved aspartic acid residues essential for cleavage of prepilin substrates and secretion of their ectodomains [61]. While the two essential aspartates reside outside predicted TMDs and the substrate cleavage site is likewise outside the membrane, TFPP provided important early support for presenilins as proteases, even though they are evolutionarily unrelated membrane proteins. This support was strengthened by the recognition that both TFPP and presenilins contain a GXGD active-site motif [62].

More convincing corroborating evidence came with the discovery of human signal peptide peptidase (SPP), responsible for the removal of remnant signal peptides left in the membrane by the action signal peptidase. Conversion of a peptidomimetic SPP inhibitor into a biotinylated affinity reagent led to the specific labeling of a presenilin homolog (PSH) [63]. Such PSHs, distantly related to presenilins, had been identified through searching of genomic databases and were found even in microbial organisms [64]. Cloning and expression in yeast of the identified target of the SPP inhibitor and isolation of yeast microsomes revealed that this human PSH had protease activity on its own, unlike the presenilins [63].

The evidence for presenilins as proteases was compelling. However, the absence of proteolytic activity on their own, the assembly into high-molecular-weight complexes, and conversion into stable NTF/CTF heterodimers together indicated that presenilins were the catalytic component of a larger γ-secretase complex [41–48]. The search for cofactors, protein partners for human presenilins, first identified a 709-residue type I transmembrane protein dubbed nicastrin by immunoprecipitation [65]. Presenilin and nicastrin were still insufficient to reconstitute γ-secretase activity, so apparently other components remained to be identified.

Genetic screening in *C. elegans* for novel components of the Notch signaling pathway led to the discovery of anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2) [66–70]. Aph-1 was predicted to be a ~25 kDa seven-TMD protein, and Pen-2 as a small (101 amino acid) protein with two predicted TMDs. Expression of all four proteins (PSEN1, nicastrin, Aph-1, and Pen-2) resulted in PSEN1 endoproteolysis to NTF and CTF and reconstitution of γ-secretase activity in mammalian cells and yeast [7–9], and all four proteins (presenilin as NTF and CTF) copurified by affinity isolation using an immobilized transition-state analog inhibitor of γ-secretase [8].

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With the identification of all the essential components of γ-secretase (Fig. 2), the question of the size and stoichiometry of the protease complex remained. Native gel electrophoresis gave varying results from 250–900 kDa [71–74], and some studies suggested the possibility of two presenilin molecules per complex [73, 75, 76]. Differential epitope labeling and co-immunoprecipitation, however, supported only one of each component in proteolytically active γ-secretase [77]. Furthermore, purification of the active protease allowed scanning transmission electron microscopy and determination of a ~230 kDa complex, again consistent with one of each component [78]. Initial elucidation of a detailed structure of the γ-secretase complex by cryo-electron microscopy (cryoEM) further confirmed a 1:1:1:1 stoichiometry [79]. Although this issue seemed settled, a more recent study has suggested the possibility that the protease may consist of two interacting 1:1:1:1 complexes [80].

An interesting mechanistic question is how TMD substrates gain access to what was presumed to be an active site sequestered inside presenilin, thereby avoiding interaction of the TMD aspartates and water with the hydrophobic lipid tails. The first clue was the copurification of an endogenous APP substrate upon isolation of γ-secretase using an immobilized transition-state analog inhibitor [81]. The substrate was capable of stably interacting with the protease even though the active site was occupied by the immobilized inhibitor, suggesting the existence of an initial substrate docking site on the outside of presenilin. Subsequent movement of the substrate TMD in whole or in part into the internal active site would allow proteolysis. Further evidence for a docking site came from enzyme inhibition kinetics [82] and designed helical peptide inhibitors based on the APP TMD [83, 84]. Conversion of these inhibitors to affinity reagents led to labeling of PS1 NTF and CTF, as seen with transition-state analog inhibitors [85]. However, the two types of inhibitors could not compete with each other, indicating distinct binding sites. Lengthening the helical peptide inhibitors from 10 to 13 amino acids resulted in competition with the transition-state analog inhibitors, suggesting that the active site and docking sites were proximal, within the length of three amino acids.

A remarkable recent study involved the systematic installation of a photoreactive phenylalanine analog into APP substrate and analyzing the labeling of γ-secretase subunits at each position [86]. Labeling was strongest in the C-terminal region of the substrate TMD, specifically at positions 42, 44, 45, and 49 (which labeled PS1 NTF) and positions 51 and 52 (which labeled PS1 CTF). This finding supports the model for substrate TMD interacting at the interface between PS1 NTF and CTF [85, 87]. Very little binding to other γ-secretase subunits were observed, although low-level labeling was seen to nicastrin with the photoreactive residue in the lumen/extracellular N-terminal region of the substrate, and to Pen-2 with the reactive residue in the juxtamembrane regions [86]. (A new report also suggests some interaction of the N-terminal region of APP substrate with nicastrin [88].) Interestingly, two FAD mutations in PS1 resulted in a shift in the labeling patterns with the photoreactive substrates [86]. This altered interaction of APP substrate with FAD-mutant γ-secretase may result in the observed altered patterns of Aβ production.

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4. Proteolytic Function and Dysfunction

Substrate requirements for γ-secretase appear to be quite broaden, with many type I integral membrane proteins serving as substrates [1–3]. However, this is generally only after lumenal/extracellular ectodomain shedding, suggesting that substrate must have a relatively short juxtamembrane region to access the γ-secretase active site. An early report using an *in vivo* genetic reporter assay in *Drosophila* with Notch as substrate concluded that presenilin-dependent processing did not depend on the specific TMD sequence [89]. Interestingly, the TMD of glycophorin A, known to dimerize as a coiled coil, did not appear to serve as a substrate, but a single point mutation that disrupts dimerization apparently allowed presenilin-dependent cleavage. The length of the extracellular domain was also found to be critical: increasing the excellular domain beyond ~50 amino acids hindered processing in a length-dependent manner. Subsequent biochemical studies have confirmed and extended these early *in vivo* findings [90, 91]. Interestingly, a substrate with a naturally short ectodomain was identified recently [92].

The lack of TMD specificity apparently allows γ-secretase to process many integral membrane protein stubs. A proteomic analysis was conducted using stable isotope labeling with amino acids in cell culture (SILAC) in the presence and absence of a γ-secretase inhibitor, revealing increases in putative membrane-bound CTF substrates [3]. Follow-up genetic and pharmacological experiments validated genuine substrates. Not all membrane protein stubs were processed by γ-secretase, suggesting that non-substrates may have inhibitory domains, a concept supported by a study of the role of the juxtamembrane region in APP processing [93]. Over 90 substrates for γ-secretase have been identified (selected examples listed in Fig. 1). Indeed, one of its biological functions may be to clear out membrane protein stubs, leading to γ-secretase being dubbed "the proteasome of the membrane"[4]. However, inhibition or knockout of γ-secretase does not generally lead to toxicity in cell culture, suggesting that general removal of protein stubs is not essential or that other means of removal exists.

In whole organisms, however, γ-secretase is an essential enzyme. Genetic knockout of γ-secretase is embryonic lethal, due to inhibition of the Notch1 signaling pathway [15, 16]. Beyond development, inhibition of γ-secretase in human clinical trials also resulted in severe toxic effects such as gastrointestinal bleeding, immunosuppression, and skin lesions, likewise due to inhibition of Notch signaling needed for regulation of cell differentiation [94, 95]. The central importance of Notch signaling often masks effects of inhibiting the proteolysis of other critical γ-secretase substrates. These include N- and E-cadherin (cell-cell adhesion) [17, 18], ErbB4 (receptor tyrosine kinase signaling) [19], p75 NTR (neurotrophin signaling) [96], and the β2 subunit of the voltage-gated sodium channel (cell-cell adhesion and migration) [97]. The essential nature of γ-secretase notwithstanding, PSEN2 can be knocked out in mice, resulting in only a mild pulmonary phenotype with age [98]. PSEN2 is incorporated into distinct γ-secretase complexes [99], and while there are few FAD-linked PSEN2 mutations, these also can increase Aβ42 production (e.g., [32, 33]).

Proteolytic processing by γ-secretase has been most comprehensively studied with APP substrate. Sequence analysis revealed that the secreted Aβ peptides and AICD proteolytic

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products were not generated from a single proteolytic event. The AICD-generating cut site was dubbed the ε cleavage site, 3–4 residues into the TMD on the cytosolic side [100–104]. Longer AICD fragments complementary to the secreted Aβ peptides were not observed. The identification of Aβ peptides produced by so-called ζ cleavage, Aβ45 and Aβ46, were then identified [11, 105]. The expression in mammalian cells of Aβ48 and Aβ49 peptides complementary to the AICD products—fused to a signal peptide for membrane insertion—led to production of Aβ40 and Aβ42 that could be blocked by inhibition of γ-secretase, suggesting a precursor-product relationship [10]. Aβ49 gave primarily Aβ40, while the poorly expressed Aβ48 gave equal but low levels of Aβ40 and Aβ42.

Taken together, these findings led to the hypothesis that γ-secretase initially cleaves at the ε site to release AICD and generate Aβ48 and Aβ49, which are then trimmed generally every three amino acids along two pathways: Aβ49→Aβ46→Aβ43→Aβ40 and Aβ48→Aβ45→Aβ42→Aβ38 (Fig. 3) [11]. The detection of the small peptide by-products by mass spectrometry from enzyme assays supported this model [13], which was subsequently validated using purified γ-secretase preparations and synthetic Aβ45 and Aβ48 (converted primarily to Aβ42 over Aβ40) and Aβ46 and Aβ49 (converted primarily to Aβ40 over Aβ42) [14]. Whether all γ-secretase substrates are processed in this manner is not known, although proteolysis of the Notch1 TMD likewise leads to generation of NICD with only 3–4 TMD residues [38] and secreted "Nβ" peptides of varying lengths [106] that cannot be explained by a single proteolytic event. Moreover, PSEN1 autoproteolysis also apparently involves tripeptide trimming [107], and both Notch and PSEN1 processing are affected by PSEN1 FAD mutations [106, 107].

As noted earlier, FAD mutations in the presenilins were found to increase the proportion of the much more aggregation-prone Aβ42 over the predominant secreted product Aβ40. Thus, Aβ42 has been presumed as the pathogenic form of Aβ and the major focus of studies aimed at determining the role of Aβ in Alzheimer's disease. However, this "toxic gain-of-function" hypothesis to explain the pathogenicity of PSEN FAD mutations has been called into question, including in a recent report showing that many PSEN1 FAD mutations do not increase the Aβ42/Aβ40 ratio in isolated enzyme reactions [108]. (Note: This study itself has been called into question [109].) An alternative hypothesis posits that presenilin FAD mutations lead to a loss of function [110, 111], pointing to how most FAD mutations reduce NICD release from Notch receptors [112] and that two mutations (L435F, C410Y) cause complete loss of production of secreted Aβ [113].

The loss-of-function hypothesis, however, has inconsistencies as well: (1) Not all PSEN1 FAD mutations result in a reduction of ε-site cleavage [114, 115], (2) no FAD mutations lead to nonsense-mediated decay (NMD); remarkably, NMD mutations in γ-secretase components lead to familial acne inversa, not neurodegeneration [116] (3) C410Y and L435F PSEN1 do in fact support Aβ production, and L435F primarily produces Aβ43 that deposits in the form of cerebral plaques in mutation carriers [117, 118]. Lost in the debate over gain-of-function versus loss-of-function is that PSEN/γ-secretase has multiple proteolytic functions. A comprehensive analysis of all proteolytic products—including small peptide byproducts—generated by PSEN FAD-mutant protease complexes should help resolve this question. At present, evidence suggests that PSEN1 FAD mutations cause a

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reduction in the carboxypeptidase (aka trimming) function of γ-secretase, rather than in its endoproteolytic function [14, 114, 115, 119, 120].

5. Structure of γ-secretase

Perhaps the most remarkable breakthrough in the study of γ-secretase has been structure elucidation. Advances in cryo-EM technology have revolutionized structural biology, allowing the determination of high-resolution structures of large complexes [121]. The application of cutting-edge cryo-EM approaches led to the first detailed structure of the γ-secretase complex, showing a horseshoe-shaped arrangement of its 19 total TMDs and the location and orientation of the nicastrin ectodomain [79]. A follow-up study provided full assignment of the TMDs, revealing that the active site resides just within the convex side of the horseshoe-shaped arrangement (Fig. 4) [122]. Jutting out over the active site was the nicastrin ectodomain, apparently preventing the approach of substrates with long lumenal/ extracellular domains. The new structure was consistent with a concurrent biochemical study demonstrating that shorter substrate luminal/extracellular domains were processed faster, that the substrate TMD is sufficient for high-affinity binding and processing by γ-secretase, and that reducing the disulfide bonds in nicastrin allowed longer substrates to be processed [91]. The new structure was also consistent with previous biochemical reports on the arrangement of the subunits within the complex [72, 123]. Unexpectedly, TMD 1 of Pen-2 was found not to pass through the membrane; rather, it appears to dip in and out of the membrane (This finding was concurrently confirmed through cellular/biochemical experiments [124]). Pen-2 interacted directly with TMD 4 of PSEN1, consistent with mutagenesis studies [125, 126].

Another cryo-EM study employed an image classification technique to reveal three different conformations of the protease complex [127]. In one of these structures, an unidentified electron density consistent with a transmembrane helix was observed, and the position of this helix within the PSEN1 subunit strongly suggested that endogenous membrane proteins copurified with the γ-secretase complex. The helical-shaped electron density disappeared as it approached the catalytic aspartates, and none of the identified copurifying proteins were previously known to be substrates. Nevertheless, the observed density provided evidence for where substrate might bind to the protease. Moreover, comparison with the other two observed conformations suggested that TMD 2 and the cytosolic end of TMD 6 undergo substantial changes upon substrate binding. In this same study, the protease complex was also analyzed in the presence of an inhibitor called DAPT. The enzyme assumed a similar conformation to that seen with the unidentified helical density (putative endogenous substrate that copurified), suggesting that DAPT inhibits γ-secretase by inducing the protease into a conformation normally formed upon substrate binding.

Most recently, two structures of γ-secretase bound to recombinant substrates, APP [88] and Notch [128], have been reported (shown with APP in Fig. 5). To capture these substrates with γ-secretase, the investigators mutated one of the active site aspartates to alanine, thereby disabling protease function, and cysteine-crosslinked the substrate to Loop 1 of PSEN1. The N-terminal region of substrate was α-helical and in a closely similar location inside PSEN1 as the unidentified helical density in the earlier study [127]. However, for both

APP and Notch substrates, as the helical TMD of substrate approaches the active site, unwinding and extension occurs, forming a β-sheet with the cytosolic end PSEN1 TMD 7. Although the enzyme is in an inactivated state due to mutation of a catalytic aspartate, the new structures revealed that substrate unwinding and extension is critical for setting up intramembrane proteolysis. Remarkably, the majority of FAD mutations in PSEN1 appear to interact with the substrate-binding cavity, either directly or indirectly, an important clue to the pathogenic mechanism of these dominant mutations.

6. Perspective

Since the initial discovery of presenilin as a novel membrane-embedded aspartyl protease, substantial progress has been made in understanding the structure, mechanism, and biology of the γ-secretase complex. Molecular biology and chemical biology have been critical to this progress, and most recently structural biology has provided atomic-level details of the protease in several different conformations, include inhibitor-bound and substrate-bound states. Nevetheless, important steps in substrate recognition and processive proteolysis by γ-secretase remain to be elucidated. Also critical is deciphering the effects of FAD mutations—both in presenilins and APP—on the various proteolytic events carried out by the enzyme complex. It seems more than merely coincidental that FAD mutations have been found only in the substrate and enzyme that directly produce Aβ. A comprehensive understanding of the effects of FAD mutations on the processing of APP substrate by γ-secretase should expedite the unraveling of molecular details of pathogenesis and the discovery of therapeutic agents for the prevention and treatment of Alzheimer's disease.

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Abbreviations:

Aβ	amyloid β-peptide
APP	amyloid precursor protein
AICD	APP intracellular domain
Aph-1	anterior pharynx-defective 1
cryoEM	cryo-electron microscopy
CTF	C-terminal fragment
FAD	familial Alzheimer's disease
NTF	N-terminal fragment
Pen-2	presenilin enhancer 2
PSEN	presenilin
PSH	presenilin homolog

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SPP	signal peptide peptidase
TMD	transmembrane domain
TFPPs	type 4 prepilin peptidases

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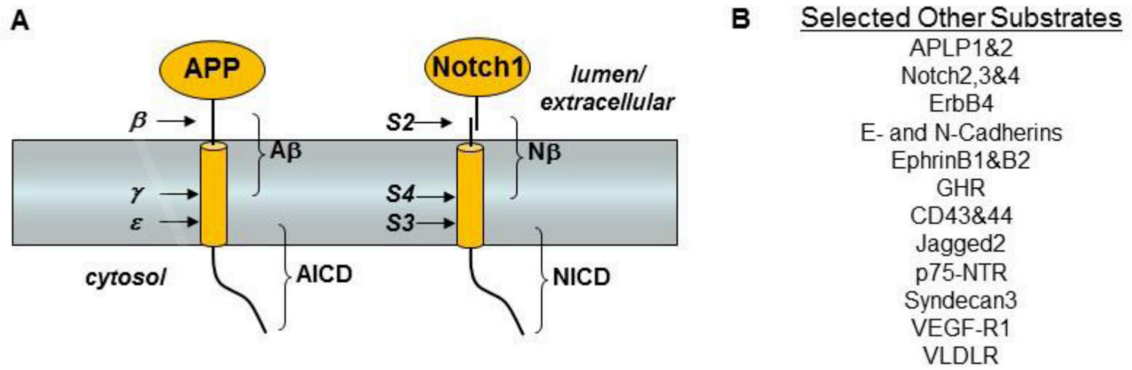

Figure 1.

Substrates for γ-secretase. (**A**) Proteolytic processing of APP and Notch. The ectodomain of APP is first shed by β-secretase. Alternatively, APP can be cut within the Aβ region by α-secretases (not shown). The remaining membrane-associated stub is cleaved at least twice in the transmembrane region, at the γ site to produce Aβ and at the ε site to produce the intracellular domain (AICD). The Notch receptor is first processed in the secretory pathway at the S1 site by furin (not shown) to produce a heterodimer. Upon activation by ligand, this heterodimer is first cleaved at the S2 site by metalloproteases to shed the ectodomain, and the remaining membrane-associated stub is cleaved within the transmembrane domain at the S3 and S4 sites. The transmembrane cleavage events are carried out by the presenilin-containing γ-secretase complex. (**B**) A partial list of the ~90 other type I integral membrane proteins cleaved within their transmembrane regions by γ-secretase.

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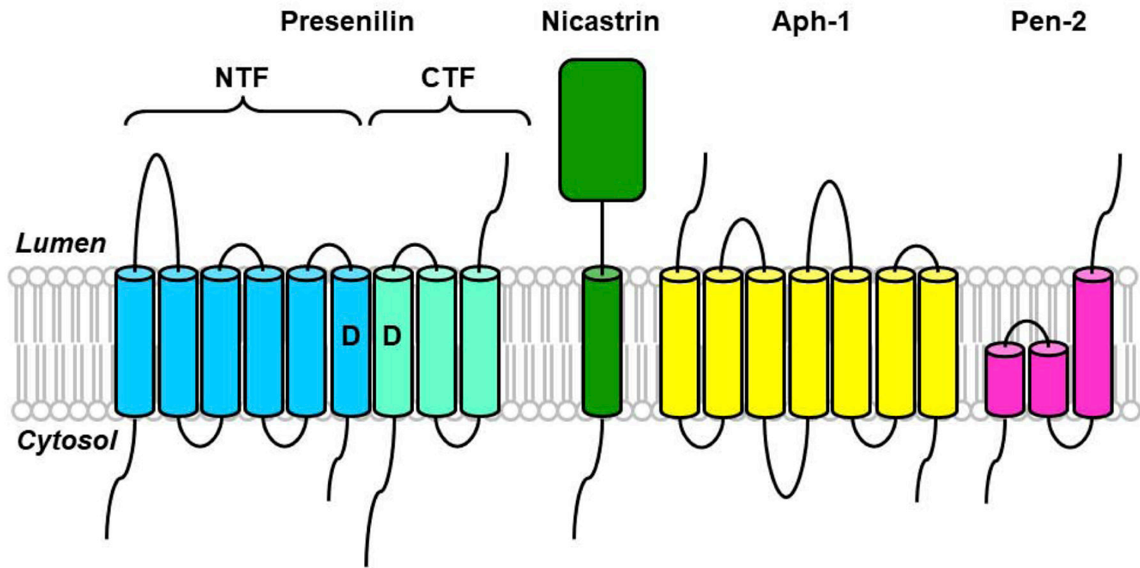

Figure 2.

Components and assembly of the γ-secretase complex. γ-Secretase is composed of four different integral membrane proteins: presenilin, nicastrin, Aph-1, and Pen-2. Presenilin undergoes endoproteolysis into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain associated. Two conserved aspartates within adjacent transmembrane domains are essential for both presenilin endoproteolysis and γ-secretase activity.

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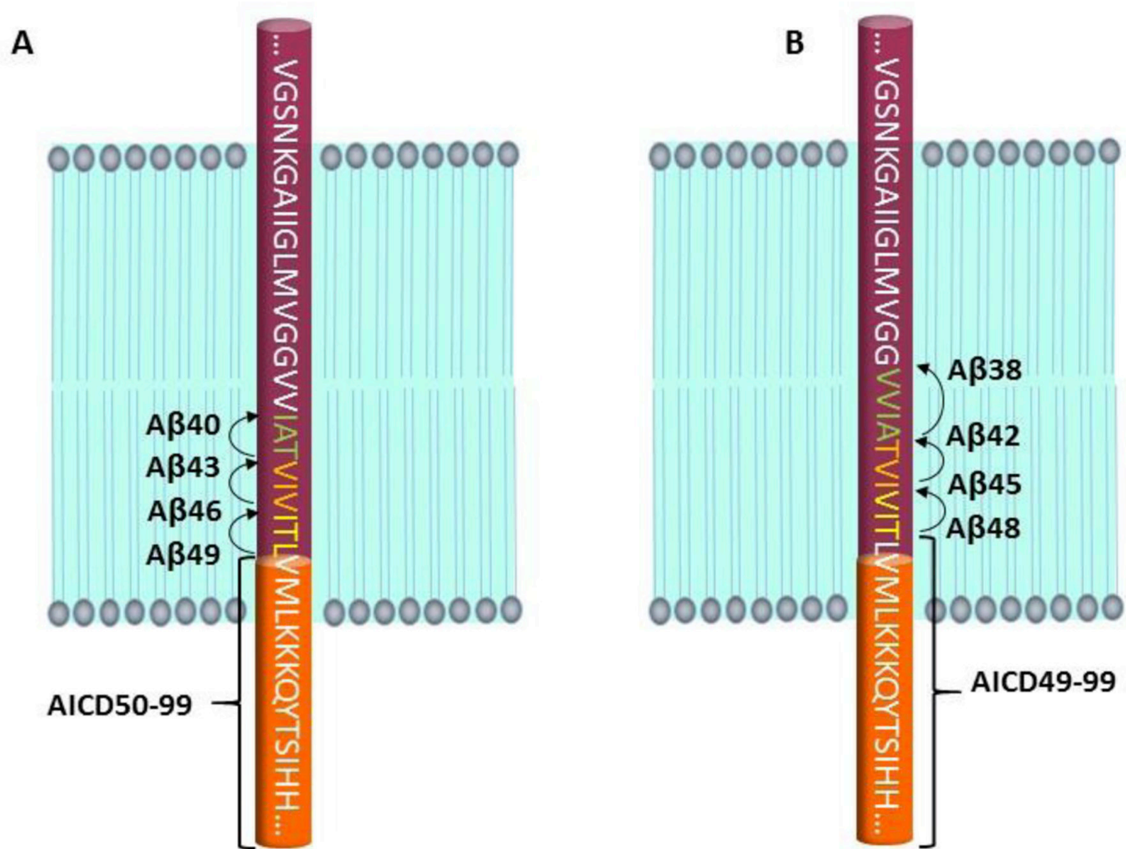

Figure 3.

Processive proteolysis of the APP transmembrane domain by γ-secretase. An endoproteolytic activity of the enzyme cleaves at the ε site within the transmembrane domain close to the membrane-cytosol interface to give long Aβ peptides Aβ48 and Aβ49 and the APP intracellular domain (AICD). The carboxypeptidase activity of γ-secretase then trims Aβ48 and Aβ49 in 3–4 amino acid increments along two pathways: (**A**) Aβ49→Aβ46→Aβ43→Aβ40 and (**B**) Aβ48→Aβ45→Aβ42→Aβ38.

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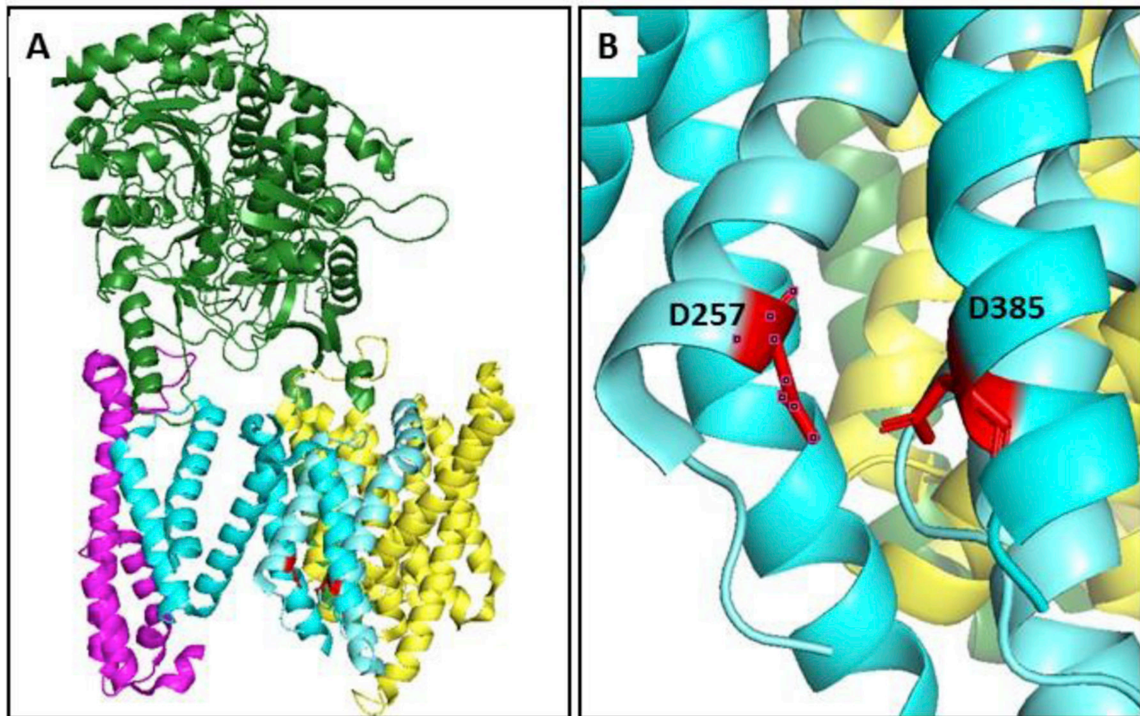

Figure 4.

First detailed structure of the γ-secretase complex determined by cryo-EM, single-particle analysis, and image reconstruction. (**A**) Nicastrin: green; Aph-1: yellow; Pen-2: magenta; PSEN1: cyan (NTF) and aquamarine (CTF), with catalytic aspartates in red. PSEN1 TMD 2 was not resolved. (**B**) The active site on PSEN1, with D257 in TMD 6 and D385 in TMD 7 in close proximity. PDB: 5A63.

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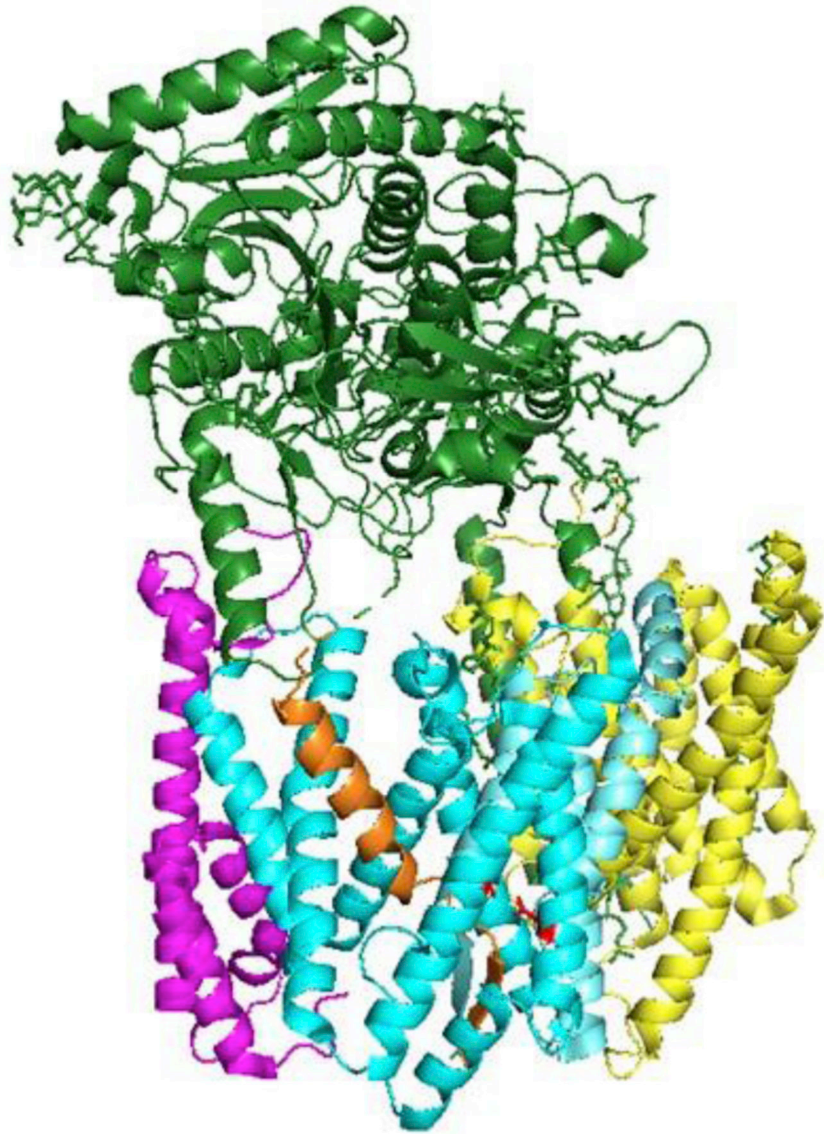

Figure 5.

γ-Secretase bound to APP-derived substrate. APP substrate is located inside PSEN1. The substrate TMD assumes a β-strand conformation near the cytoplasmic side as it interacts with the active site. PSEN1 TMD 2 is now visible (foreground), as is the cytoplasmic side of TMD 6 (cf. Figure 4). PDB: 6IYC.