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Assembly, Trafficking and Function of γ-Secretase

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Alzheimer's disease · Amyloid-β peptide · γ-Secretase · Presenilin

Abstract
γ-Secretase catalyzes the final cleavage of the β-amyloid precursor protein to generate amyloid-β peptide, the principal component of amyloid plaques in the brains of patients suffering from Alzheimer's disease. Here, we review the identification of γ-secretase as a protease complex and its assembly and trafficking to its site(s) of cellular function. In reconstitution experiments, γ-secretase was found to be composed of four integral membrane proteins, presenilin (PS), nicastrin (NCT), PEN-2 and APH-1 that are essential and sufficient for γ-secretase activity. PS, which serves as a catalytic subunit of γ-secretase, was identified as a prototypic member of novel aspartyl proteases of the GxGD type. In human cells, γ-secretase could be further defined as a heterogeneous activity consisting of distinct complexes that are composed of PS1 or PS2 and APH-1a or APH-1b homologues together with NCT and PEN-2. Using green fluorescent protein as a reporter we localized PS and γ-secretase activity at the plasma membrane and endosomes. Investigation of γ-secretase complex assembly in knockdown and knockout cells of the individual subunits allowed us to develop a model of complex assembly in which NCT and APH-1 first stabilize PS before PEN-2 assembles as the last component. Furthermore, we could map domains in PS and PEN-2 that govern assembly and trafficking of the complex. Finally, Rer1 was identified as a PEN-2-binding protein that serves a role as an auxiliary factor for γ-secretase complex assembly.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder in industrial countries. Neuropathological hallmarks of AD are the deposition of the 40–42 amino acid amyloid-β peptide (Aβ40, Aβ42) as senile plaques and of hyperphosphorylated tau as neurofibrillary tangles. In their accompanying articles, Mandelkow and Mandelkow review in detail the cellular mechanisms causing neurofibrillary tangle formation and its impact on AD. Aβ42 is highly neurotoxic and believed to be the culprit of the disease, which initiates a cascade of pathological events that ultimately lead to dementia [1]. Aβ is generated by proteolytic processing of the β-amyloid precursor protein (APP), a type I transmembrane protein [2]. APP is first processed by β-secretase, which leaves a 99 amino acid C-terminal fragment (C99) in the membrane. Aβ is subsequently liberated into the extracellular space by γ-secretase cleavage of C99 within the membrane. This cleavage also releases the

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APP intracellular domain (AICD) into the cytosol. A competing nonamyloidogenic processing pathway of APP involves cleavage by α-secretase within the Aβ domain, generating an 83 amino acid membrane bound C-terminal fragment (C83). Subsequent cleavage of C83 by γ-secretase causes the liberation of the nonamyloidogenic peptide p3. α-Secretase cleavage thus precludes the formation of Aβ. Fahrenholz and Postina [this issue, pp. 255–261] and Lichtenthaler [this issue, pp. 262–269] describe this pathway in detail. A central question of AD research of nearly two decades has been the elucidation of the identity of the secretases which generate Aβ. In particular γ-secretase, which is the focus of this review, has been an enigmatic enzyme for a long time [3].

Identification of γ-Secretase

Ten years ago, a handful of mutations causing early-onset familial AD (FAD) were discovered and mapped to two novel genes located on chromosomes 1 and 14 [4–7]. The responsible genes, termed presenilin 1 (PS1) and presenilin 2 (PS2) were predicted to encode two homologous ~50-kDa polytopic membrane proteins, that according to recent models consist of 9 transmembrane domains (TMDs) [8–10]. Both are endoproteolytically cleaved between TMDs 6 and 7 into an N-terminal and C-terminal fragment (NTF, CTF) [11]. Although PSs lacked obvious functional homology to other proteins when they were first described, the identification of *Caenorhabditis elegans* SEL-12 as PS homologue [12] strongly implicated PSs in the Notch signaling pathway required for cell differentiation [see also the review article by Smialowska and Baumeister in this issue, pp. 227–232]. Like FAD-associated mutations found earlier in the C-terminal end of the APP TMD, the FAD-associated mutations in PS1 and PS2 increased the levels of Aβ42 [13]. The number of mutations found increased rapidly to more than 150 in 2005, including 144 PS1 mutations. PS1 is therefore the major gene responsible for early-onset FAD. The finding that FAD-associated mutants shifted the cleavage specificity of γ-secretase towards Aβ42 formation suggested that PSs might be modulators of γ-secretase causing a gain of function. On the other hand, knockout of the mouse PS1 gene caused a severe reduction of total Aβ generation [14]. Furthermore, cells derived from PS1 knockout mice showed an accumulation of the C-terminal fragments of APP, while leaving α- and β-secretase cleavage unchanged. Taken together, these data indicated that PS is intimately associated with γ-secretase activity and hinted to the possibility that PSs were either γ-secretase itself or alternatively an essential co-factor of it.

Considerable evidence for the first hypothesis that PS is identical with γ-secretase was obtained when mutagenesis of either of two aspartate residues of PS1 with an unusual location in TMD6 and 7 caused the same γ-secretase loss of function phenotype as found for the PS1 gene deletion [15]. Moreover, when these aspartates were mutated, PS accumulated as uncleaved full-length holoprotein, suggesting the possibility that PS autoactivates itself. These findings were consistent with the earlier observation that γ-secretase is an aspartyl protease [16]. Further support for the hypothesis that PS is identical with γ-secretase was obtained when we observed loss of γ-secretase function coupled with a deficiency of PS endoproteolysis upon mutation of the corresponding aspartate residues in PS2 and zebrafish PS1, thus demonstrating that the critical PS aspartates were functionally conserved during evolution [17, 18]. Another piece of evidence was the finding that crosslinkable γ-secretase inhibitors designed to mimic the transition-state of an aspartyl protease mechanism were found to covalently bind the PS NTF and CTF [19, 20]. While these observations were consistent with PS being a candidate aspartyl protease identical with γ-secretase, the lack of any homology to other aspartyl proteases and the lack of the canonical D(T/S) G(T/S) active motif of aspartyl proteases remained in apparent contrast to the hypothesis. This puzzling issue was resolved when we identified G384 in PS1 as an essential residue of PS function [21]. This residue is part of a highly conserved small GxGD motif that includes the critical aspartate in TMD7 of PS. Moreover, we identified this motif in the bacterial type 4 prepilin peptidase (TFPP) family, polytopic proteases that function as leader peptidases. Similar to PSs, the TFPPs contained two critical aspartate residues, directly adjacent to the TMD boundaries, required for their proteolytic function [22]. The latest piece of evidence for a proteolytic function of PS was the subsequent identification of signal peptide peptidase (SPP) and its homologues, the SPP-like (SPPL) proteases [23]. Like TFPPs and PSs, SPP and SPPLs contain the GxGD active site motif [24]. In addition, all three families contain a short conserved PxL motif at the C-terminus. Despite these conserved regions, no further homologies are found. Interestingly, PS and SPP differ in their orientation of the active sites towards the substrate. While PS cleaves substrates like APP in type I orientation, SPP [23] and SPPLs [Fluhrer et al., submitted] use type II membrane proteins as substrates. Taken together, PS was identified as a founding member of novel polytopic aspartyl

proteases of the GxGD type [25]. For a detailed description of SPP and SPPLs, we refer the reader to the accompanying review by Haffner and Haass.

While these findings strongly suggested that PS might indeed be identical with the long-sought γ-secretase and provided compelling evidence that PS has to be regarded as a novel aspartyl protease, other findings indicated that it might not fulfill the γ-secretase function alone. In fact, data by others and us suggested that PS resides in a high molecular weight (HMW) complex [26–28], and indeed it was subsequently shown that γ-secretase activity was present in an HMW complex as PS-dependent activity [29]. Moreover, overexpression of PS neither led to an increase in the NTF and CTF [30] nor to increased γ-secretase activity. This finding suggested that PS expression is regulated by the presence of other (limiting) factors, which together with PS assemble into an HMW complex [30] allowing PS endoproteolysis. Consistent with this observation, we found that excess PS holoprotein that fails to become processed into its stable fragments is rapidly degraded by the proteasome [31].

Using an immunoaffinity isolation procedure, the type I membrane glycoprotein nicastrin (NCT) was the first PS-binding partner identified [32]. In addition, screening for Notch pathway components in *C. elegans* identified two novel candidate PS partner proteins besides NCT, the polytopic membrane proteins PEN-2 and APH-1 [33, 34]. Coimmunoprecipitation studies revealed that PEN-2 and APH-1 are indeed in association with PS and NCT [35, 36]. Strikingly, when we coexpressed these four proteins in baker's yeast, which does not contain homologues of these proteins and has no endogenous γ-secretase activity, γ-secretase activity towards an APP-based substrate was fully reconstituted [37]. γ-Secretase activity required the coexpression of all four components and was not observed when either one of the four components was lacking. Moreover, reconstitution of γ-secretase activity was associated with PS endoproteolysis and was found to be dependent on biologically active PS. These experiments demonstrated that γ-secretase is a complex of four core components that are necessary and sufficient for the activity of the γ-secretase enzyme. Similar results were obtained when the four components were overexpressed in mammalian cells [38–40]. γ-Secretase activity was significantly enhanced when all four proteins were coexpressed, suggesting a reconstitution of the enzyme. These findings also demonstrated that NCT, APH-1 and PEN-2 were the elusive limiting factors for PS expression.

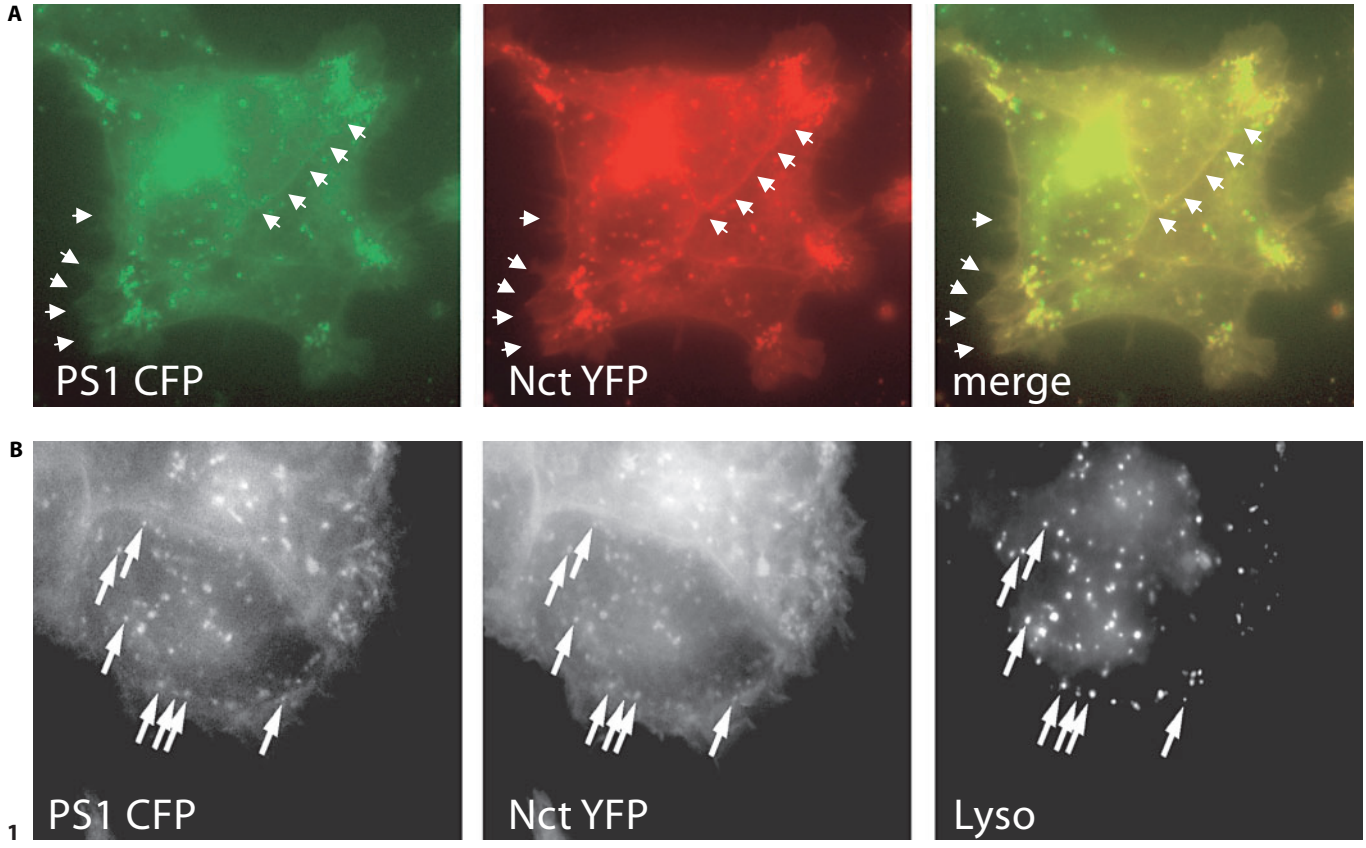
Like for PSs, two homologues of APH-1 were identified in mammalian cells, APH-1a and APH-1b, with APH-1a occurring in two splice variants differing in their C-termini [33, 36]. In a coimmunoprecipitation analysis, we found that PS1 and PS2 and the APH-1 homologues/splice variants are contained in separate γ-secretase complexes in human cells [35, 41]. Thus, the term γ-secretase reflects a heterogeneous activity in human cells that consists of several distinct complexes depending on the respective tissue expression of the core components. Similar findings were obtained in rodents [42].

Localization of γ-Secretase and Its Cellular Site(s) of Activity

Shortly after the initial identification of PS1, its intracellular localization was determined to be mostly in the endoplasmic reticulum (ER) [43]. In contrast, presumed sites of γ-secretase activity ranged from the ER to the Golgi, TGN, secretory vesicles, plasma membrane (PM) and endosomes/lysosomes [for a discussion, see 44]. Subsequent analysis in neurons and other cell types led to the proposal of the 'spatial paradox', a term coined by Annaert and De Strooper [45], that referred to the apparent discrepancy in the localization of PS in the ER and the sites of γ-secretase activity proposed to be in later compartments of the secretory pathway.

To clarify the localization of PS, we used green fluorescent protein (GFP) as a reporter for live cell staining. GFP-tagged PS1 (PS1-GFP) was shown to be fully functional in all aspects tested. PS1-GFP replaced endogenous PS1/2, was incorporated into an HMW complex and rescued γ-secretase activity in PS1/2–/– cells. Using total internal reflection microscopy and cell surface biotinylation we could show that small but significant amounts of PS1-GFP were localized at the PM [46]. Moreover, PS1 bound to NCT could be detected at the PM, showing that it is indeed complex-associated PS, which is at the PM [46]. In living cells expressing PS1-CFP and NCT-YFP, both subunits colocalize at the PM and in endosomes/lysosomes, supporting the idea that fully assembled complexes leave the ER and reach later compartments of the secretory pathway (fig. 1). Others subsequently confirmed these results by the demonstration that all four γ-secretase complex components are localized in an active form at the PM [47] and in lysosomes [48].

To determine the localization of γ-secretase activity with a novel approach, we again made use of GFP. A γ-secretase substrate, the APP C-terminal stub (C99), was tagged C-terminally with GFP (C99-GFP) [49]. When stably expressed in cells, this substrate is efficiently cleaved


A
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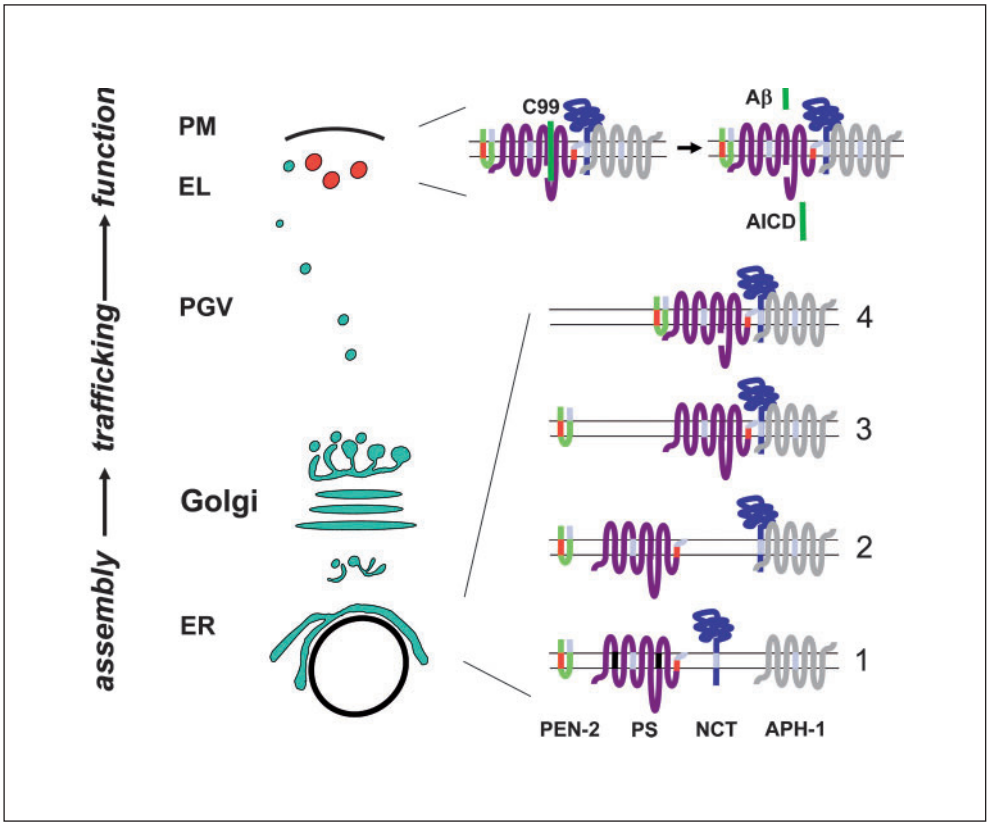
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by γ-secretase, resulting in a diffuse cytoplasmic GFP staining. When γ-secretase is blocked using specific inhibitors, C99-GFP is no longer cleaved and remains membrane associated, highlighting the compartment where it resides. To determine the sites of γ-secretase activity, biosynthetic transport was inhibited at defined steps along the secretory pathway. The rationale behind that study was that if γ-secretase cleavage occurs in the compartment where transport was blocked, C99-GFP would be cleaved and the same diffuse GFP fluorescence as in control conditions would be observed. If however γ-secretase is not active in the compartment where transport is blocked, C99-GFP would remain membrane bound and highlight the respective organelle. Transport of C99-GFP was inhibited at the level of the ER, the Golgi and the TGN. In all cases, membrane-bound C99-GFP accumulated in the respective organelle, indicating that there is no substantial γ-secretase activity in the ER, the Golgi and the TGN. When exocytosis of post-Golgi vesicles was inhibited, bright fluorescent vesicles accumulated below the PM of C99-GFP-expressing cells, demonstrating that C99-GFP is not cleaved before it reaches the PM. In contrast, when endocytosis was blocked in C99-GFP-expressing cells, GFP staining was weak and diffuse, showing that γ-secretase cleaves C99-GFP at the PM. Whether γ-secretase is in addition active in endosomes could not be tested using this system. Taken together, our data show that γ-secretase activity is localized to the PM and/or endosomes [49].

Fig. 1. Live cell microscopy of HEK293 cells stably expressing PS1-CFP and NCT-YFP. In addition, these cells express siRNA against endogenous NCT. PS1-CFP and NCT-YFP are fully functional and assemble into γ-secretase complexes. **A** Two-color microscopy of living cells shows a high degree of colocalization of PS1-CFP and NCT-YFP at the PM (arrowheads) and in vesicular structures. Note that some vesicles appear green or red only in the merged image due to vesicular movement during image acquisition. **B** Three-color live cell microscopy demonstrates that the vesicular structures seen in **A** are endosomes/lysosomes, as demonstrated by labeling with lysotracker (arrows).

Fig. 2. Assembly of γ-secretase. (1) Unassembled subunits of the γ-secretase complex are retained in the ER by specific ER retention signals (red bars). (2) APH-1 and NCT form a first assembly intermediate, which then stabilizes PS holoprotein (3). (4) Finally PEN-2 joins the complex, endoproteolysis of PS and conformational change of NCT take place and the fully assembled complex is exported from the ER through the Golgi via post-Golgi vesicles (PGV, green vesicles) to the PM. Protein-protein interacting domains are depicted in light blue. At the PM and/or in endosomes/lysosomes (EL, red vesicles) the complex cleaves C99 (green) to release Aβ and AICD.

Assembly of the γ-Secretase Complex

How do the components assemble to build a γ-secretase complex? Insight into this question was largely obtained from experiments using knockouts and knockdowns of the individual subunits in cultured cells. We found that knockdown of NCT and APH-1 by RNAi was accompanied by a strong reduction of the PS fragment levels [41, 50]. Furthermore, when PEN-2 expression was knocked down, the PS holoprotein accumulated in an unprocessed form [51]. In contrast, the knockout of PS was accompanied with decreased PEN-2 levels, while levels of NCT and APH-1 remained largely unchanged [35, 52]. Interestingly, NCT accumulated in its immature form, suggesting that the complex cannot exit the ER in the absence of PS [50]. Furthermore, we could show that the NCT ectodomain undergoes a conformational change during the assembly process [52]. Taken together, these results and the data from other investigators [36, 38–40, 53, 54] suggested a model for stepwise assembly of γ-secretase complex(es) [41]. First, NCT and APH-1 form an initial stable scaffold for the PS holoprotein, which becomes stabilized by the interaction with the NCT/APH-1 assembly intermediate. Next, association of PEN-2 to this trimeric assembly intermediate triggers the endoproteolytic cleavage of the PS holoprotein (fig. 2). Finally, on the molecular level we and others could identify TMD4 of APH-1 [55, 56], the C-terminus of PEN-2 [51, 57–59], the TMD of NCT [60, 61] and the PS1 C-terminus [62, 63] as essential domains for functional γ-secretase complex assembly. The NCT TMD and the PS1 C-terminus interact directly with each other [62]. Recent data also suggest direct interactions of the PEN-2 TMD1 with the PS1 TMD4 [64, 65].

Are there specific signals or domains within the γ-secretase subunits, which govern the assembly of the γ-secretase complex? Analogous to ion channels and cell surface receptors, which are frequently composed of several subunits, we and others found that γ-secretase is assembled in the ER [59, 66]. In the case of ion channels and cell surface receptors, it is known that control mechanisms ensure that only fully assembled complexes leave the ER, while unassembled subunits are retained/retrieved by specific retention/retrieval signals. We hypothesized that similar mechanisms steer the correct assembly and export of γ-secretase out of the ER. Indeed, using reporter proteins to study cell surface transport, we could identify ER retention/retrieval signals in two γ-secretase subunits, PS1 and PEN-2. The ER retention/retrieval signal in PS1 is located in the C-terminus and

includes the PALP-motif [62]. The ER-retention signal of PS1 is probably embedded in the membrane, as a number of groups recently showed that the hydrophobic part of the PS1 C-terminus spans the membrane. This suggests a topology with nine TMDs for PS [8–10]. In the case of PEN-2, the retention/retrieval signal is located in TMD1 and involves a critical asparagine [Kaether et al., submitted]. The molecular machinery recognizing these signals in mammals is unknown. In yeast, a protein called Rer1p was shown to retrieve unassembled subunits of several complexes to the ER. Retrieval was based on retrieval signals located in transmembrane segments and involved polar or charged amino acids surrounded by hydrophobic amino acids [67]. The human orthologue, Rer1, is a 23-kDa protein with four TMDs that can complement a yeast *RER1* gene deletion strain [68]. We could show with reporter protein assays and deglycosylation experiments that human Rer1 is involved in the retention/retrieval of PEN-2. In addition, we showed that the mammalian Rer1 binds directly to unassembled PEN-2. Binding depends on a critical asparagine in the first TMD of PEN-2. Furthermore, overexpression of Rer1 stabilizes PEN-2 and enhances maturation of immature NCT, indicating an enhanced rate of complex formation. These data support the idea that PEN-2 is rate limiting for γ-secretase complex formation and identify Rer1 as a possible auxiliary factor for γ-secretase complex assembly [Kaether et al., submitted].

Cellular Function of γ-Secretase

PSs have been implicated in the Notch signaling pathway, which is required for cell differentiation during development and adulthood, due to the discovery of the *C. elegans* PS homologue SEL-12 as a key component of this signaling pathway [12]. Consistent with this finding, knockout of PS and the other γ-secretase subunits in mice causes phenotypes with Notch-like embryonic development deficiency [69]. In addition, we found in collaboration with Baumeister's group that FAD-associated PS mutations largely fail to rescue the Notch deficiency phenotype of *sel-12* mutant worms, whereas PS active site mutations do not rescue at all [17, 21, 70–72]. Notch is a cell surface receptor with type I membrane topology, which is processed in a very similar manner like APP [69]. Following cleavage of the Notch ectodomain at the cell surface, we found that γ-secretase cleaves the resultant C-terminal Notch membrane fragment to release Nβ, an Aβ-like peptide, into the extracellular space and the Notch intracellular domain (NICD) into the cytosol [73]. The NICD translocates to the nucleus, where it functions as a transcriptional regulator of target genes required for cell differentiation. Thus, enabling Notch signaling is a major function of γ-secretase. However, besides the two substrates APP and Notch, a rapidly increasing number of other substrates of γ-secretase have been discovered recently. Among these substrates are CD44 and LRP, both of which were identified in laboratories from the priority program [74, 75]. These substrates have little in common except that they are all type I transmembrane proteins that need to undergo ectodomain shedding that removes the bulk of their extracellular domains to become substrates for the enzyme [76]. While some of the ICDs liberated might have a function in nuclear signal transduction similar to the NICD, a more general function of γ-secretase may be the removal of membrane stubs of type I membrane proteins after ectodomain shedding. Interestingly, recent data suggest that NCT serves as a γ-secretase substrate sensor probably by measuring the length of the ectodomains of type I transmembrane proteins [77].

Conclusions

Together with the work in a number of other laboratories worldwide, the γ-secretase research within the priority program 1085 of the DFG has considerably expanded our knowledge about a long-sought, enigmatic enzyme that was known to be responsible for the final step in the biogenesis of Aβ. We could clarify the identity of γ-secretase by demonstrating that γ-secretase is a complex consisting of four subunits that are required for its activity. We further developed an understanding of how the γ-secretase complex assembles and how the complex traffics through the secretory pathway to its functional site(s). Finally, the identification of PS, the catalytic subunit of γ-secretase, as a prototype of novel aspartyl protease families and the elucidation of signals that govern γ-secretase complex assembly also contributed to the opening of new research fields in cell biology.

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