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Regulation of γ-Secretase Activity in Alzheimer's Disease

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ABSTRACT: The γ-secretase complex is an intramembrane aspartyl protease that cleaves its substrates along their transmembrane regions. Sequential proteolytic processing of amyloid precursor protein by β- and γ-secretase produces amyloid β-peptides, which are the major components of amyloid plaques in the brains of Alzheimer's disease patients. The γ-secretase complex is therefore believed to be critical in the pathogenesis of Alzheimer's disease. Here we review the range of factors found to affect the nature and degree of γ-secretase complex activity; these include γ-secretase complex assembly and activation, the integral regulatory subunit CD147, transient or weak binding partners, the levels of cholesterol and sphingolipids in cell membranes, and inflammatory cytokines. Integrated knowledge of the molecular mechanisms supporting the actions of these factors is expected to lead to a comprehensive understanding of the functional regulation of the γ-secretase complex, and this, in turn, should facilitate the development of novel therapeutic strategies for the treatment of Alzheimer's disease.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder and the most common cause of dementia. It is estimated that about 10% of individuals over age 65 and nearly half over age 85 are affected with AD (*1*). The impact of AD on individuals, families, and the health care system makes this disease one of the greatest medical, social, and economic challenges in the world. The pathological features of AD as seen in the central cortex and limbic system of the brain are extracellular amyloid plaques mainly composed of amyloid β-peptides (Aβ-peptides) and intracellular neurofibrillary tangles (NFTs) made of hyperphosphorylated microtubule-associated protein tau. Although it is generally believed that both Aβ-peptides and tau are intimately involved in causing AD (*2*), it appears that Aβ-peptide aggregation provides the initial insult (*3*).

AD occurs in two general forms, familial and sporadic. Familial AD (FAD) is a rare form of AD, affecting about five percent of AD patients. All FAD is early onset, meaning the disease develops before age 60, as early as the 30s and

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[1] Abbreviations: AD, Alzheimer's disease; Aβ-peptides, amyloid β-peptides; NFTs, neurofibrillary tangles; FAD, familial Alzheimer's disease; APP, amyloid precursor protein; PS1, presenilin-1; PS2, presenilin-2; ApoE, apolipoprotein E; TM, transmembrane; AICD, APP intracellular domain; S2P, site 2 protease; SPP, signal peptide peptidase; Nct, nicastrin; APH-1, anterior pharynx defective-1; Pen-2, presenilin enhancer-2; NTF, N-terminal fragment; CTF, C-terminal fragment; CD, cluster differentiation antigen; DCC, deleted in colorectal cancer; LRP, low-density lipoprotein receptor-related protein; GSK, glycogen synthase kinase; PLD1, phospholipase D1; TMP21, 21 kDa transmembrane-trafficking protein; PSIG, presenilin-independent γ-secretase; TMD, transmembrane domain; ER, endoplasmic reticulum; TGN, trans-Golgi network; HMGR, hydroxymethylglutaryl-CoA reductase; SM, sphingomyelin; nSMase, neutral sphingomyelinase; DRM, detergent-resistant membrane; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-1β, interleukin-1β; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase; DDM, *n*-dodecyl-β-D-maltoside; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Brij 96, polyoxyethylene 10 oleoyl ether; siRNA, small interfering RNA.

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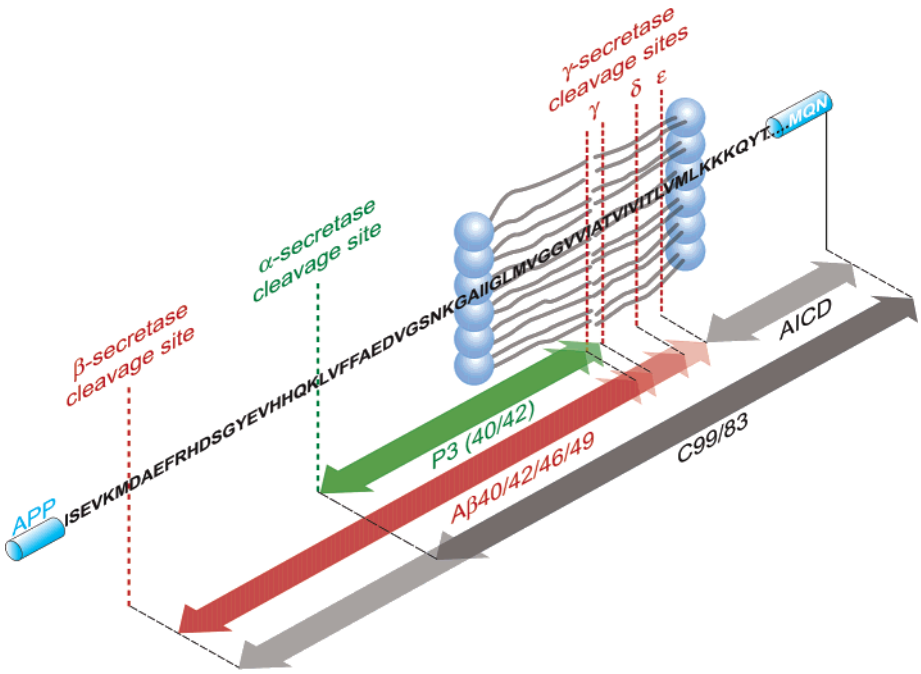

FIGURE 1: APP proteolytic processing. APP is initially cleaved by α- or *β*-secretases, generating membrane-bound C-terminal fragment C83 or C99. *γ*-Secretase can then cleave C83 along its TM region to produce the non-neurotoxic peptide P3 (40/42) and C99 at one of several sites to produce a range of neurotoxic A*β*-peptides, cutting at *γ*-cleavage site to produce A*β*40/42, at *δ*-cleavage site to produce A*β*46, or at *ϵ*-cleavage site to produce A*β*49 and APP intracellular fragment (AICD). The APP polypeptide is depicted here with its N-terminal end on the left-hand side of the figure. The extracellular space is also on the left-hand side of the lipid bilayer, while the cytoplasmic space is on the right-hand side.

40s. FAD has obvious genetic etiology; various mutations in the genes coding for presenilin-1 (PS1), presenilin-2 (PS2), and amyloid precursor protein (APP) have been found in FAD families (*4*−*8*). *In vivo* and *in vitro* studies involving pathogenic gene mutations of PS1, PS2, and APP show a resultant increase in the production of A*β*-peptides, especially A*β*42 (*9*−*12*). These findings led to the identification of PS as a component of *γ*-secretase and established the connection between *γ*-secretase activity and the pathogenesis of AD (*13*, *14*).

The majority of AD cases are sporadic late-onset, usually developing after age 65. Unlike FAD, late-onset AD has no definite genetic etiology. The ApoE (apolipoprotein E) gene, which codes for a protein that helps transport cholesterol in the blood, has been identified as a risk factor because inheritance of the ApoE e4 allele appears to increase the risk of developing late-onset AD (*15*). There may be other currently unknown genetic linkers associated with late-onset AD, but it appears that increasing age is the greatest risk factor. Late-onset AD shows virtually identical pathological features as early onset FAD, yet it has not been found to be linked to PS or APP gene mutations. It is reasonable to expect that defects in the regulation of *γ*-secretase activity play a major role in the pathogenesis of late-onset AD.

APP, a type I transmembrane (TM) glycoprotein believed to play a role in neuroprotection, synaptic transmission, signal transduction, and axonal transport (*16*−*18*), is a target of α-, *β*-, and *γ*-secretase processing (Figure 1). Cleavage of APP begins at one of two pathways, by α-secretase to produce soluble APP_{α} and a membrane-bound C-terminal fragment C83 or by *β*-secretase to generate soluble APP_{β} and the membrane-bound C-terminal fragment C99 (*19*). α-Secretase precludes while *β*-secretase facilitates the generation of A*β*-peptides. The cellular regulation of these two alternative pathways has not been fully characterized. α-Secretase cleavage of APP has been found to take place in the plasma membrane, while *β*-secretase cleavage occurs mainly within endosomes/lysosomes that have the acidic pH necessary for maximal *β*-secretase activity (*19*). However, *β*-secretase may act at other sites within the cell, because A*β*-peptides have been reported to be produced in a variety of locations including the ER, TGN, and cell surface (*19*, *20*). Protein kinase A mediated intracellular signaling, membrane cholesterol composition, and APP endocytosis have been reported to participate in the regulation of α- and *β*-secretase cleavage of APP (*19*).

γ-Secretase cleaves α- and *β*-secretase products C83 and C99 within their TM regions to produce the non-neurotoxic peptide P3 and several neurotoxic A*β*-peptides. The majority of A*β*-peptides produced are 40 amino acids long (A*β*40), whereas A*β*-peptides 42 amino acids long (A*β*42) are a substantially less abundant species, typically produced at about one-tenth the level of A*β*40. A*β*42 is more hydrophobic and the predominant A*β*-peptide found in amyloid plaques and is believed to be a pivotal player in the pathogenesis of AD (*21*, *22*).

In addition to *γ*-cleavage at the 40 and 42 sites, *δ*- and *ϵ*-cleavage sites at residues 46 and 49, respectively, have also been observed (Figure 1) (*23*−*25*). The APP intracellular domain (AICD) is produced by the *ϵ*-cleavage and may be involved in the regulation of transcription (*26*, *27*). Due to its role in performing the final proteolytic step in the APP processing cascade to produce A*β*-peptides, the mechanism of *γ*-secretase-mediated proteolysis has been of great interest.

The *γ*-secretase complex is a member of the intramembrane protease superfamily having an unusual aspartyl protease activity in which proteolytic events occur within lipid bilayers (Figure 2) (*13*); the superfamily also includes

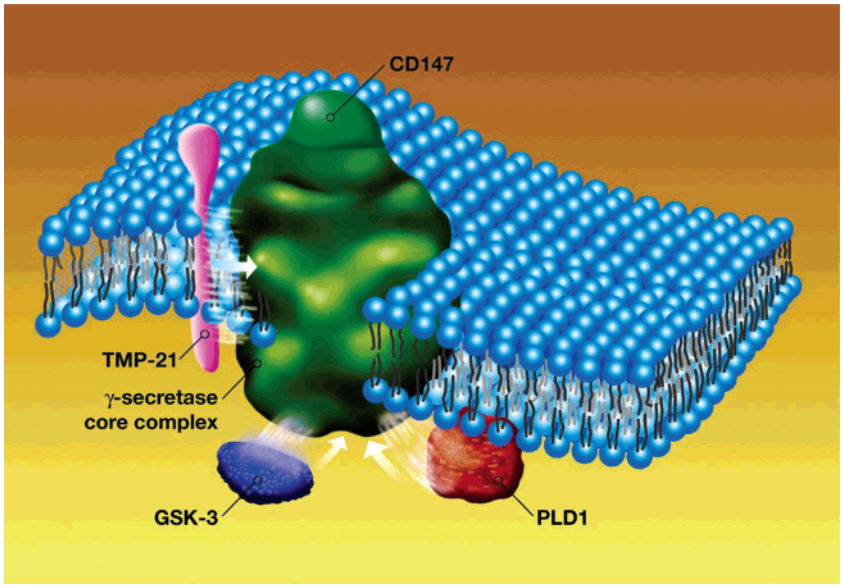

FIGURE 2: γ-Secretase complex and binding partners. PS, Nct, APH-1, and Pen-2 are assembled to form the enzymatic core complex. CD147 is an integral regulatory subunit of the native complex. TMP21, PLD1, and GSK3 have been found to modulate γ-secretase activity and transiently or weakly bind to the complex; transient interactions with the complex are depicted by white arrows.

the family of S2P metalloproteases (*28*, *29*), the signal peptide peptidase (SPP) aspartyl protease (*30*), and the rhomboid serine protease family (*31*, *32*). Biochemical and genetic studies have demonstrated that presenilin (PS), nicastrin (Nct), anterior pharynx defective-1 (APH-1), and presenilin enhancer-2 (Pen-2) form the enzymatically active core of the γ-secretase complex (Figure 2) (*33*, *34*). PS provides the proteolytic active site, which by itself is inactive. Upon assembly of the enzymatic core complex subunits, PS is autocatalytically processed to yield an N-terminal fragment (NTF) and C-terminal fragment (CTF), which in turn establishes proteolytic activity (*35*−*37*). Substantial experimental evidence demonstrates that these four components are necessary and sufficient for obtaining γ-secretase activity (*38*−*41*). In addition to APP, the γ-secretase complex processes numerous other type I TM proteins including fragments of Notch, E-cadherin, N-cadherin, CD44, DCC, ErbB4, LRP, and nectin-1, which are involved in many physiological and pathological functions (*42*−*50*).

Purification of endogenous γ-secretase complexes from detergent-solubilized HeLa cell membranes shows that they contain the four components of the enzymatic core and an additional integral subunit, the membrane protein CD147 (Figure 2). CD147 was found to have a regulatory effect on γ-secretase activity (*51*). Transient or weak binding partners of the γ-secretase complex, such as glycogen synthase kinase-3 (GSK-3), phospholipase D1 (PLD1), and TMP21, have been reported (Figure 2) (*52*−*55*). While the presence of these binding partners has also been found to affect the activity of γ-secretase, none of these proteins have been detected in chromatographically purified samples of the complex.

Several factors or processes have been reported to affect the nature and degree of γ-secretase activity; these include γ-secretase complex assembly and activation, the integral regulatory subunit CD147, transient or weak binding partners, inflammatory cytokines, and cholesterol and sphingolipid levels, which may affect the proteolytic microenvironment of the γ-secretase complex. In addition to the action of the PS-dependent γ-secretase complex in the process of Aβ-peptide production, a PS-independent γ-secretase (PSIG)-like activity has been reported by several research groups, observed in studies with PS1-, PS2-, or PS1- and PS2-deficient cell lines, to contribute to the production and accumulation of intraneuronal Aβ42 (*56*−*58*). The identity of PSIG enzymes has not yet been determined, and whether such enzymes impact the pathogenesis of AD, especially late-onset AD, remains to be elucidated. In this review, the factors affecting γ-secretase activity and the subsequent generation of Aβ-peptides are discussed in greater detail.

Components and Activation of the γ-Secretase Complex

Enzymatic Core

The initial indication of a γ-secretase complex maturation process was found in the proteolytic processing of PS (*35*). At that time, PS was the only known member of γ-secretase, found to be synthesized as a 50 kDa holoprotein and subsequently endoproteolyzed by an unknown enzyme, named presenilinase, to generate a 30 kDa NTF and a 20 kDa CTF (*35*, *36*). PS is present as an NTF−CTF heterodimer in the mature active form of the γ-secretase complex (*37*). It was proposed that there are factors that are responsible for controlling PS endoproteolysis and stabilizing NTF−CTF heterodimers (*35*, *36*). Subsequent genetic and biochemical studies have identified additional components of the γ-secretase complex, Nct, APH-1, and Pen-2, and demonstrated that these components directly participate in PS processing and subsequent activation of the γ-secretase complex (*38*, *50*, *59*−*64*).

The connection of PS to AD was discovered through genetic studies on FAD family members (*5*−*8*). Pathogenic mutations of PS-1 and PS-2 were found to be responsible for the overproduction of the most amyloidogenic form of amyloid peptide (Aβ42/43) (*9*−*12*). Further investigations demonstrated that PS itself is an integral membrane aspartyl protease and that its two conserved aspartate residues are critical for γ-secretase activity (*13*, *14*). Several different topography models for PS have been proposed, suggesting

a secondary structure of either 6-TM, 7-TM, 8-TM, or 9-TM domains (*65*−*70*). The 8-TM structural model, where the N-terminal and C-terminal ends as well as the hydrophilic loop between TM6 and TM7 are located at the cytoplasmic face of the plasma membrane, has been the most widely accepted. A 9-TM topography model has been most recently proposed (*69*, *70*).

The early evidence that Nct is an essential component of the γ-secretase complex came from RNA interference experiments in *Caenorhabditis elegans*, where deletion of the Nct gene produced an embryonic lethal phenotype, highly reminiscent of those produced by reduction of gene activity involved in the Notch signaling pathway or by reduction of PS expression (*50*). Nct is a ~110 kDa glycosylated type I TM protein with a large N-terminal extracellular domain and a short (20 residues) intracellular domain. Two forms of Nct have been observed, termed "immature" and "mature", differing in their extent of glycosylation. The fully glycosylated form appears to be the only form able to bind to PS and associate with the γ-secretase complex (*59*, *71*); however, it has also been suggested that glycosylation may not be required for Nct function (*72*). Nct has been shown to be required for PS endoproteolysis and is essential for γ-secretase-mediated cleavage of both APP and Notch (*50*). The large extracellular domain of Nct was found to interact with the N-terminal region of γ-secretase substrates possibly aiding in the recruitment of substrates into γ-secretase complex active sites (*73*). The free N-terminal NH_2 of the substrate is important in this process since it was demonstrated that N-terminal methylation of APP C-terminal fragment (C99) substrates could block γ-secretase cleavage (*73*). However, small fluoregenic peptides that contain APP and Notch TM γ-secretase cleavage regions, but not a free N-terminal NH_2, can still be cleaved by the γ-secretase complex *in vitro* (*74*, *75*). The TM domain (TMD) of Nct, especially residues of the N-terminal region of the TMD, has been found to be required for complex assembly through experiments in which chimeras involving the TMD (i.e., NCT−TMD and ADAM-10-TMD) were studied (*76*).

Genetic screening for mutations in *C. elegans* that affected Notch function, PS activity, or both led to the identification of two additional membrane proteins, ~30 kDa APH-1 and 12 kDa Pen-2, that were found to play critical roles in γ-secretase activity (*61*, *77*). APH-1 and Pen-2 were subsequently shown to be integral components of the γ-secretase complex through coimmunoprecipitation and RNA interference experiments (*38*, *61*−*64*). APH-1 is predicted to be a 7-TM protein and has been found to form a subcomplex with Nct and stabilize the presence of the PS holoprotein within the complex (*38*, *60*, *78*, *79*). Pen-2, a membrane protein with two putative TM helices, has been suggested to facilitate the endoproteolysis of PS, the stabilization of the resulting N- and C-terminal fragments, and the subsequent activation of the γ-secretase core complex (*38*−*40*, *60*, *64*, *80*). Massey et al. have recently reported that ubiquilin-1 and ubiquilin-2 are also involved in the regulation of PS endoproteolytic processing, because they found that overexpression of ubiquilin-1 or -2 decreased the levels of PS NTF and CTF and that inhibition of proteasome activity resulted in decreased PS NTF and CTF production (*81*). Based on these observations, it was suggested that the proteasome might be directly responsible for PS proteolysis; however, an alternative interpretation, implicating an indirect role for the proteosome involving other proteins or pathways, cannot be ruled out at this time.

When expressed in yeast, the γ-secretase complex subunits PS, Nct, APH-1, and Pen-2 were found to be necessary and sufficient to produce an enzymatic core complex with γ-secretase activity (*39*). A model of the assembly pathway for these four members of the enzymatic core complex has been proposed (*33*). In this assembly model, APH-1 and partially glycosylated ("immature") Nct form a stable subcomplex early during assembly; this step is believed to occur in the ER. Subsequently, the PS holoprotein joins APH-1 and Nct to form a trimeric intermediate complex (*60*, *78*). Completion of Nct glycosylation in the Golgi/trans-Golgi network and entry of Pen-2 into the maturing complex promote PS endoproteolysis and formation of an active γ-secretase core complex (*38*−*40*, *60*, *78*). It should be noted that this model only describes the steps leading to enzymatic core complex formation and does not include steps involving the incorporation of integral regulatory subunit, CD147, or interaction with transient/weak binding partners, such as TMP21.

Regulation of the γ-Secretase Complex by Subunit CD147

Membrane protein CD147 was recently identified as an integral subunit of the native γ-secretase complex through the purification and analysis of endogenous complexes from HeLa cell membranes (*51*). The complexes were purified to a homogeneous state solubilized with the highly effective detergents FOS-CHOLINE-12 or DDM. Coimmunoprecipitation experiments conducted on both the purified complex and cell membranes from various cell lines confirmed that CD147 is an integral component of the γ-secretase complex. It is important to note that, in such experiments, the type of detergent used is critical in assessing whether a membrane protein is an integral member of a particular complex. Use of detergents with weak solubilizing characteristics, such as CHAPSO and Triton X-100, is typically not effective in obtaining homogeneous populations of membrane proteins because such detergents can preserve weak associations of even functionally unrelated proteins mediated through unsolubilized cell membrane fragments. However, the use of mild detergents can be a useful tool in identifying transient or weak binding partners. For example, caveolin (*82*) and two β1 integrins (*83*) were found to be weakly associated with CD147 when a mild detergent such as Brij 96 was used to solubilize cell membranes prior to immunoprecipitation. Even substrates of the γ-secretase complex (C99- and C83-βAPP) have been found to coimmunoprecipitate with complex subunit Nct when sample membranes were solubilized with the mild detergents digitonin or NP-40 (*50*). However, such associations are seldom maintained when samples are isolated using more aggressive detergents, such as FOS-CHOLINE-12.

Through CD147 RNA interference and overexpression investigations, the presence of CD147 in endogenous γ-secretase complexes was found to down-modulate the production of Aβ-peptides (*51*); its effect on the processing of other γ-secretase substrates, such as Notch, remains to be determined. CD147 is a glycosylated type I TM protein with a molecular weight of about 50 kDa. It is predicted to have a

short cytoplasmic domain about 40 amino acids long, a putative 25 amino acids long TM region, and a large extracellular region consisting primarily of two immunoglobulin-like (Ig-like) domains (*84*). Sequence motif analysis indicates a high probability of phosphorylation at the two C-terminal serines, possibly involving protein kinase A or protein kinase B (Akt). Unlike other type I TM proteins, such as Nct and the substrates of γ-secretase, the putative TM domain of CD147 contains a highly conserved glutamic acid, postulated to be located within the hydrophobic core of the cell membrane. However, charged residues are not commonly found in the TM regions of proteins that span the membrane only once; the placement of a charged residue in the middle of the lipid bilayer would be highly energetically unfavorable. This structural feature suggests that CD147 associates with other membrane proteins to exist in an energetically stable state. Interestingly, although the number of amino acids assigned to this domain is sufficient for establishing a TM helix, secondary structure in this segment is predicted to be non-helical and rich in β structure. Such secondary structure might place this charged residue away from the hydrophobic core of the membrane. It should also be noted that the γ-secretase subunit PS contains two negatively charged residues, aspartic acids, within its putative TM region. The recently determined structure of the intramembrane protease, GlpG, and the low-resolution structure of the γ-secretase complex suggest that a water-filled pocket may be the means for energetically accommodating such charged residues (*85*, *86*). Elucidation of how CD147 interacts with other members of the endogenous γ-secretase complex, especially when it is thought to accommodate a charged residue within its transmembrane region, and the molecular mechanism by which CD147 exerts its effect on γ-secretase activity will clearly require the high-resolution structure of the native complex.

Yeast two-hybrid studies revealed an association of cyclophilin A with CD147 through which binding was found to stimulate an increase in intracellular calcium and the phosphorylation of extracellular signal-regulated kinase (ERK) (*87*). Of potential relevance is a very recent report that suggested that differential ERK phosphorylation in skin fibroblasts induced by the inflammatory mediator bradykinin could be a peripheral biomarker for clinical diagnosis of AD (*88*). However, it is unknown whether this difference in phosphorylation levels is a causative factor in or a consequence of the disease.

Other Regulatory Binding Partners of γ-Secretase Complex

In addition to the membrane protein CD147, several other proteins, including glycogen synthase kinase-3 (GSK-3), phospholipase D1 (PLD1), and TMP21 have been reported to physically and functionally associate with γ-secretase complexes (Figure 2) (*52*−*55*). These proteins have been found to modulate γ-secretase activity but, unlike CD147, are characterized as transient or weak binding partners of the γ-secretase complex since they are not found in chromatographically purified preparations of the complex (*41*, *51*).

Soluble protein GSK-3 has been found to phosphorylate tau at many sites found phosphorylated on tau in NFTs in AD brain (*89*−*91*). It was reported that GSK-3β and tau physically bind to PS1 along the same region, residues 250−298, which suggested that PS1 may regulate the interaction of tau with GSK-3β (*52*). Site-directed mutagenesis indicated that PS1 residues Ser353, Ser357, and Ser397 are GSK-3β targets; substitution of Ser353, Ser357, or both with alanine greatly reduced the ability of PS1 to associate with β-catenin, while substitution of Ser397 with alanine increased the levels of PS1 CTF but not PS1 NTF or holoprotein (*53*). Based on these findings, it was suggested that GSK-3β might also participate in regulating the formation of the active PS NTF−CTF. In addition to the potential effect on tau pathology in AD, GSK-3 appears to mediate the regulation of Aβ-peptide production, although differing results have been reported (*52*, *53*, *92*, *93*). GSK-3, including GSK-3α (approximate molecular weight 51 kDa) and GSK-3β (approximate molecular weight 47 kDa), participates in the Wnt signaling pathway (*94*) and is implicated in the hormonal control of several regulatory proteins including glycogen synthase, Myb proto-oncogene protein (MYB), and the transcription factor AP-1 (JUN) (*95*). Lithium chloride has been reported to reduce Aβ-peptide production through its ability to inhibit GSK-3β activity (*96*, *97*). In contrast to this notion, the reduction of GSK-3β has been reported to increase Aβ-peptide production, whereas the reduction of GSK3α levels was found to decrease Aβ-peptide production (*92*), yet GSK-3α and GSK-3β are 97% amino acid identical within their kinase domains. Further complicating the picture are the results of a recent study that demonstrated that lithium chloride can increase the production of Aβ-peptides independently from its role in GSK-3 inhibition but rather through an increase in β-secretase activity (*93*).

PLD1 is a 124 kDa phospholipase peripherally associated with cell membranes through its own lipid anchors and found to play a critical role in numerous cellular pathways, including signal transduction, membrane trafficking, and the regulation of mitosis. Furthermore, PLD1 may be also involved in the regulation of perinuclear intravesicular membrane traffic (*98*). Coimmunoprecipitation and immunocytochemistry staining experiments using wild-type and PS1/PS2-deficient embryonic stem (ES) cells solubilized with the rather mild detergent NP40 demonstrated that PS1 interacts with and recruits PLD1 to the Golgi/trans-Golgi network (TGN) through its hydrophilic loop region (*54*). Overexpression of wild-type PLD1 or catalytically inactive PLD1 (K898R) was shown to decrease the production of Aβ-peptides and the association of γ-secretase components. Down-regulation of PLD1 by RNA interference increased the production of Aβ-peptides and the association of γ-secretase components. Based on these observations, it was suggested that the regulation of γ-secretase activity by PLD1 occurs through protein−protein interactions, that is, affecting the assembly of γ-secretase components, and is independent of phospholipase catalytic activity. However, no interactions were detected between PLD1 and other γ-secretase components such as Nct, APH-1, and Pen-2 in this study (*54*).

TMP21, a 25 kDa type I membrane protein and a member of the p24 cargo protein family, is involved in vesicular protein trafficking between the ER and the Golgi complex and was found to be a γ-secretase complex interacting partner through coimmunoprecipitation of complexes from CHAPSO-solubilized membranes using antibody against the PS1

N-terminus (*55*). The γ-secretase complexes obtained from this procedure had a mass of more than 650 kDa, substantially larger than the purified active complexes chromatographically prepared by others (*41*, *51*), suggesting that additional components may exist in these complexes. In support of this possibility, CHAPSO is known to be a mild detergent able to preserve weak associations of even functionally unrelated proteins. RNA interference-based knock-down of TMP21 expression was found to elevate the production of Aβ peptides without any changes in the levels of endogenous PS1, Nct, APH-1, and Pen-2, suggesting that TMP21 modulates γ-secretase activity by a method other than by altering γ-secretase component expression levels, complex assembly, and stability. It was also found that the suppression of TMP21 expression by siRNA had no discernible effect on ϵ-cleavage activity as measured by the production of intracellular domains (ICDs) of APP, Notch, and the cadherins, leading to the suggestion that the modulating effect of TMP21 on γ-secretase activity is specific to γ-site cleavage.

Additional Factors Affecting γ-Secretase Activity

Cholesterol Metabolism and the Lipid Bilayer Environment. An association between cholesterol levels and the development of AD was suggested in the early 1990s (*99*). Since then, an increasing amount of evidence derived from genetic, epidemiological and biochemical studies has reinforced the notion of a link between cholesterol and the development of AD. In addition to age, a risk factor for late-onset AD appears to be the presence of the e4 allele of the ApoE gene, which codes for an apolipoprotein involved in cholesterol transport (*15*). There are three common alleles of the ApoE gene: e2, e3, and e4. Among them, e3 is the most common and e4 is present in at least one copy in ∼25% of the population. It has been shown that ApoE4 can more effectively bind Aβ and promote Aβ fibril formation than the ApoE3 isoform. ApoE4 not only facilitates Aβ internalization and aggregation, it may also alter brain cholesterol homeostasis by modifying lipoprotein-particle formation (*100*, *101*). Epidemiological studies have found that high blood cholesterol levels in mid-life correlate strongly with amyloid deposition and the risk of developing AD in later life (*102*, *103*). Some clinical studies have shown that people who are treated with cholesterol-lowering drugs, known as statins, present a lower incidence of AD than the general population (*104*, *105*), although other studies have produced mixed results (*106*). Studies examining the effects of high or low cholesterol levels in both cultured cells and animal models also demonstrate a strong link between cholesterol levels and Aβ-peptide production. Experiments in which rabbits were fed a diet high in cholesterol found higher amounts of intraneuronal Aβ-peptides within the hippocampal region of their brains compared with control rabbits (*107*). Hypercholesterolemia was also shown to accelerate Aβ deposition in the brains of APP transgenic mice (*108*), while treatment of mice with cholesterol synthesis inhibitors reduced Aβ deposition (*109*). Biochemical studies on cultured cells have shown that cholesterol synthesis inhibitors and membrane cholesterol extracting drugs can induce a drastic reduction in Aβ-peptide production (*110*, *111*). Although the underlying mechanism coupling high cholesterol levels and increased Aβ-peptide production has not yet been determined, there is the possibility that cholesterol levels may be involved in the regulation of γ-secretase activity through cholesterol's effect on the physical properties of lipid bilayers. Such a notion has not been widely accepted because it is generally believed that cholesterol is unable to cross the blood−brain barrier, suggesting that neuronal cholesterol levels would be unaffected by modulating blood cholesterol levels via diet (*112*). However, this may not be completely true, because mice given a high-lipid diet have been shown to have increased cholesterol levels in the central nervous system as well as in the plasma (*108*), and brain cholesterol levels are found to increase in hypercholesterolaemic transgenic mice (*113*). Changes in the distribution of lipids and cholesterol within cell membranes are known to occur during aging (*114*), and in particular, aging has been shown to alter the transbilayer distribution of cholesterol in mice (*115*), with older mice having significantly more cholesterol in the outer leaflet of lipid bilayers than younger animals.

The production of Aβ-peptides Aβ40 and 42 may, in turn, serve to regulate cholesterol and sphingomyelin metabolism (*116*). It was observed that Aβ40 down-regulates cholesterol synthesis, through inhibition of hydroxymethylglutaryl-CoA reductase (HMGR) activity, and that Aβ42 down-regulates sphingomyelin (SM) levels through activation of the SM degrading enzyme neutral sphingomyelinase (nSMase). The results of this investigation suggest a feedback mechanism between the metabolism of cholesterol and sphingomyelin lipids, and the regulation of γ-secretase activity.

γ-Secretase complexes have been reported to localize largely within lipid rafts (*117*−*119*), which are transient membrane microdomains enriched in cholesterol and sphingolipids (*120*, *121*) and believed to act as metastable platforms allowing particular proteins to cluster together to perform various physiological processes such as intracellular membrane trafficking, cell signaling, immune response, toxin and virus entry, and apoptosis (*122*−*124*). Because of experimental limitations, the studies on γ-secretase complex localization were conducted using detergent-resistant membranes (DRMs), unsolubilized remnants of detergent-treated cell membranes. Substrates of γ-secretase complexes such as APP CTFs were also found to be enriched in DRMs, while other substrates such as the CTFs of Notch1, Jagged2, DCC, and N-cadherin were found to be significantly excluded from them (*125*). The detergent most commonly used to identify proteins residing in DRMs has been Triton X-100, but other mild detergents have also been used, and studies of DRM-localized proteins have frequently yielded diverse results, depending on the detergent used (*126*). The terms lipid rafts and DRMs are often used interchangeably, despite the fact that they have different origins and conceptual meanings. Nevertheless, based on the results of DRM studies, it has been suggested that γ-secretase activity takes place predominantly within lipid rafts (*117*−*119*). Furthermore, there are indications that there may be developmental differences in the membrane location of γ-secretase complexes, which has led to a proposal that initial residence of γ-secretase complexes in nonraft membrane regions allows for the proteolysis of a diverse range of substrates during embryonic development, whereas the subsequent translocation of the γ-secretase complex to lipid raft regions in adults facilitates the continued processing of APP, while limiting processing of other substrates, such as Notch1 (*126*). Regardless of

whether γ-secretase complexes are located in raft or nonraft regions of cell membranes, the composition and physical properties of lipids within γ-secretase complex resident regions may help modulate of γ-secretase activity, potentially affecting the functional conformation of the complex and even substrate, influencing substrate selection and the distribution of cleavage sites.

Cytokines. Chronic inflammation is one of the characteristic histopathological features of the AD brain. The correlation of inflammation and accumulated Aβ-peptides has been extensively investigated (*127*−*129*). For example, aggregated Aβ-peptides have been found to trigger the release of cytokines from monocytic cells (*127*), and Aβ-peptides can bind to complement component C1q, triggering the classical complement cascade further increasing its cytotoxic effects (*128*, *129*). Experimental evidence also indicates that, in AD brains, Aβ-peptides stimulate astrocytes to produce inflammatory factors such as cytokines, complement components, and acute phase proteins (*127*−*129*).

Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β), have been reported to stimulate γ-secretase activity and increase the production of Aβ-peptides (*130*−*133*). A marked increase in the levels of Aβ40 and Aβ42 was observed in primary astrocytes and astrocytoma cells following stimulation with a combination of IFN-γ and TNF-α or IFN-γ and IL-1β (*131*, *132*), suggesting that inflammatory cytokines could modulate the processing of APP through cytokine-derived signaling pathways. Another study demonstrated that platelet-derived growth factor (PDGF) could enhance β- and γ-secretase-mediated proteolysis of APP through a Src−Rac-dependent pathway (*133*), indicating that endogenous brain Aβ levels could be affected by a variety of inflammatory molecules. The effect of cytokines on regulating γ-secretase activity was also investigated using HEK293 cells, which were stably cotransfected with a Gal4-driven luciferase reporter gene and Gal4/VP16-tagged C99 (C99−GV), the immediate substrate of γ-secretase (*130*). This study showed that INF-γ, IL-1β, and TNF-α could specifically stimulate γ-secretase activity in the production of Aβ-peptides. The mechanisms coupling activation of various cytokine pathways with the elevation of γ-secretase activity are not yet known, and their elucidation will continue to be an active research goal.

Presenilin-Independent γ-Secretase Activity (PSIG). Based on experiments with cells in which expression of PS1, PS2, or both had been eliminated, results were obtained by several groups suggesting the existence of enzymes not dependent on PS yet having γ-secretase activity (*56*−*58*). Armogida et al. (2001) first reported that PS1 and PS2 double knock-out mouse fibroblasts were still able to generate both intracellular and secreted Aβ-peptides (*56*). Subsequently, a report was published describing observations that the production of intracellular Aβ42 occurred in PS1 and PS2 knock-out primary neurons and PS1/PS2 double knock-out stem cells and that cleavage of APP CTFs by a PS-independent γ-secretase (PSIG) takes place within the early secretory compartments, ER/IC (*57*). Recently, an *in vitro* activity assay study using PS1/PS2 double knock-out BD cell membranes also found that Aβ40 and Aβ42 could be produced and that this proteolytic process could be inhibited by the general aspartyl protease inhibitor pepstatin A, suggesting that this PSIG is an aspartyl protease (*58*). PSIG enzymes have not yet been isolated or identified. Whether the activity of PSIG affects the processing of Aβ-peptides by the γ-secretase complex, through the coupling of Aβ peptide production and cholesterol/sphingomyelin metabolism (*116*) and the effect of cholesterol levels on γ-secretase activity, is presently unclear.

Concluding Remarks

The activity of γ-secretase complex appears to be subject to multiple levels of regulation. Such regulation is essential for the coordinated proteolysis of APP and the resulting production of Aβ-peptides, which is believed to play a critical role in the pathogenesis of AD. Assembly of the enzymatic core subunits PS, Nct, APH-1, and Pen-2 is required for activation of the PS catalytic site. The integral regulatory subunit CD147, transient or weak binding partners (GSK3, PLD1, and TMP21), cholesterol and sphingolipid metabolism, and inflammatory cytokines are all factors able to modulate γ-secretase activity. The molecular mechanisms involved in the regulatory actions of these factors remain to be determined. High-resolution structural details of the γ-secretase complex followed by structurally guided mutation studies will be needed to elucidate the catalytic and regulatory processes of γ-secretase activity. Furthermore, understanding of the potential interplay between the range of regulatory factors described here will likely emerge through systems biology analysis. When obtained, in-depth knowledge of the molecular basis of γ-secetase function is expected to accelerate the development of novel therapeutic strategies and the structure-based design of drugs for the treatment of AD.

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