Intramembrane Proteolysis mediated by The γ-Secretase Complex: Nicastrin Functions as a Substrate Receptor

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The proteolytic processing of proteins within the lipid bilayer, and release of their membrane tethered biologically active fragments, fundamentally controls a growing list of cell signaling events. The γ-secretase, one of a small family of independently evolved proteases, performs this enigmatic hydrolysis of a peptide bond within the membrane. Remarkably atypical, γ-secretase activity: (1) requires a complex of proteins that include presenilin, nicastrin, Aph1, and Pen-2; (2) catalyzes the intramembrane cleavage of a broad range of substrates, regulating physiology from neurodevelopment to neurodegeneration. The aim of this thesis is to elucidate the mechanism by which the γ-secretase recognizes its substrates. I provide evidence that nicastrin, in addition to being a critical component of the complex, plays a major function in substrate recognition. The ectodomain of nicastrin binds the new amino terminus that is generated upon the prerequisite 'shedding' of substrates, thereby recruiting substrates into the γ-secretase complex. The γ-secretase complex has been traditionally viewed as a hub for signal transduction of substrates such as Notch and APP. The mechanism by which a broad range of substrates may be recognized and subsequently cleaved, as demonstrated in this thesis, supports a mutually inclusive function as a protease that has evolved to simply dispose transmembrane domains thus controlling the repertoire of a class of proteins present in the membrane.

Abbreviations

Aβ	Amyloid β-protein
AD	Alzheimer's disease
ADAM	a disintergrin and metalloprotease
AICD	APP intracellular domain
APLP	APP-like protein
APP	Amyloid precursor protein
BACE	β-site APP cleaving enzyme
CTF	C-terminal fragment
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
LIN-12	Notch orthologue in C. elegans
NCT	Nicastrin
NFT	Neurofibrillary tangle
NICD	Notch intracellular domain
NTF	N-terminal fragment
PS1	presenilin 1
PS2	presenilin 2
SEL-12	Presenilin orthologue in C. elegans
TACE	Tumor necrosis factor α converting enzyme
TM	Transmembrane

Table of Contents

The Appendix contains reprints of the following articles:

Shah S, Lee SF, Tabuchi K, Hao Y, Yu C, LaPlant Q, Ball H, Dann III C, Südhof T, Yu G. **Nicastrin Functions as a gamma-Secretase-Substrate Receptor.** Cell, 2005 Aug 12;122(3):435-447

Lee SF, **Shah S**, Yu C, Wigley WC, Li H, Lim M, Pedersen K, Han W, Thomas P, Lundkvist J, Hao YH, Yu G. **A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex.** J Biol Chem. 2004 Feb 6;279(6):4144-52.

Luo WJ, Wang H, Li H, Kim BS, **Shah S**, Lee HJ, Thinakaran G, Kim TW, Yu G, Xu H. **PEN-2 and APH-1 coordinately regulate proteolytic processing of presenilin 1.** J Biol Chem. 2003 Mar 7;278(10):7850-4.

Lee SF, **Shah S***, Li H, Yu C, Han W, Yu G. **Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch.** J Biol Chem. 2002 Nov 22;277(47):45013-9**. *co-first author**.

Shah S, Yu G. **Viewpoint; SorLa: Sorting Out APP.** Molecular Interventions; In press.

List of Figures:

Chapter 1

Introduction

Ectodomain shedding, a process where ectodomains are proteolytically released has been now attributed to a growing list of cell surface proteins. These proteins are structurally and functionally diverse and include membrane anchored growth factors, cytokine receptors, ectoenzymes, and membrane adhesion receptors. A prerequisite, ectodomain shedding event, of many group I transmembrane receptors results in a subsequent second proteolytic event within the hydrophobic lipid membrane environment. Recognition that cell surface receptors may be processed using a well conserved, widely employed, signaling mechanism fundamentally controlled by proteases inspired the term "regulated intramembrane proteolysis" (Brown, Ye et al. 2000). The regulated intramembrane proteolysis (RIP) of these substrates is believed to release the membrane tethered, biologically active, cytoplasmic domains that then may enter the nucleus as transcriptional regulators.

RIP of the various type-I membrane proteins, including the amyloid-beta precursor protein (APP) and the Notch receptor, are catalyzed by an aspartyl protease called**,** γ-secretase. The γ-secretase is a remarkably unusual enzyme. First, it requires a complex of proteins to perform its enzymatic activity. Second, it performs the intramembrane proteolysis of a substrate necessary for life in muticellular animals while cleavage of a second substrate over the lifespan of some humans contributes to memory failure. Over the years the number of substrates and the list of putative biological functions and signaling events that may be modulated by this process continue to grow. The goal of my thesis has been to contribute to the characterization of the gamma-secretase complex and elucidate how it may recognize a broad range of substrates. These

contributions have led to the understanding the major biochemical function of nicastrin. The data presented herein reveal a novel mechanism by which a broad range of diverse substrates are recognized by an unusual membrane imbedded protease complex. Supporting mutually inclusive functions of an intramembrane protease that in addition to being a signaling hub; may simply perform the disposal of transmembrane domains, thus controlling the repertoire of a class of proteins present in the membrane.

Mrs. Auguste D and Alzheimer's

"...The Disease begins insidiously with mild weakness, headaches, dizziness, and sleeplessness. Later, severe irritability and loss of memory develop. Patients complain bitterly of their symptoms. At times the Disease is associated with a sudden apoplectic attack followed by hemiplegia. Increasing loss of memory and progressive clouding of mind appear later, with sudden mood changes, fluctuating between mild euphoria and exaggerated hypchondriasis; terminally the Disease leads to stupor and child-like behavior. The features differ from patients with general paralysis by apparent calmness, by an organized behavior pattern, and by general ability of reasoning..." From the writings of Dr. Alois Alzheimer

Alzheimer's disease is a debilitating disease, one that begins in small failures in memory, which gradually become more noticeable, and eventually progress to a loss of self. A century has past since Dr. Alois Alzheimer, most famously presented his case findings of Mrs. Auguste D. At the mere age of 51 years, she was unable to remember her entire name, her husband's name or how long she had been in the hospital. On their first meeting Dr. Alzheimer would try to have her write her name. She failed several times. Exasperated, she announced, "I have lost myself"(Berrios 1990). Although Dr. Alzheimer's would be credited as the first to describe plaques and tangles covering her brain, and Alzheimer's disease would be born, revisiting history reveals that observations

of plaques and tangles association with dementia were well known at the time(Maurer, Volk et al. 1997). The novelty of his findings, which are now clear from his writings, was the striking young age at which Auguste D had first displayed her initial memory loss. A speculative conclusion of Dr. Alzheimer's initial intention of his presentation may have been to point out that senile dementia could begin at a relatively young age. Strikingly, it has been from the molecular genetic analysis of the small pedigree of those who have early onset Alzheimer's (FAD) that revealed, almost a century later, the biological culprits involved.

APP Genetics

A small percentage of patients inherit this illness as a familial autosomal-dominant (FAD) trait. Some of these traits mapped to the β-amyloid precursor protein (APP) gene, which codes for a type I single span transmembrane protein (Kang, Lemaire et al. 1987). Mutations in this gene on chromosome 21 cause FAD. APP is a precursor to amyloid β peptides (Aβ). The accumulation over a lifetime of (Aβ) deposits in senile plaques, form the hallmark lesions in the brains of patients affected by this disease. Further, evidence of a role of APP may be found in those who are born with an extra copy of chromosome 21. Only one human disease results in a more profound Alzheimer's like pathology. An extra copy of chromosome 21, with three copies of APP instead of two, results not only in (Down's syndrome), but brings with it the invariant phenotypes of Alzheimer's pathology, at even an earlier age.

A common theme among several neurodegenerative disorders such as AD, Parkinson's disease, ALS, Huntington's disease, and others is misfolding of a normally well folded protein with aggregation into lesions of the neuron. A progressive accumulation of APP gene dosage and the increase in processed unfolded Aβ early in life results in amyloid plaque formation. In the case of FAD-causing mutations in APP, assays in cell culture and from human plasma have revealed that they either increase the production of total Aβ species ($A\beta_{total}$) (Citron, Oltersdorf et al. 1992; Cai, Golde et al. 1993) or specifically tilt the favor towards a more aggregatable 42 amino acid form ($A\beta_{42}$) (Suzuki, Cheung et al. 1994). The first animal models with amyloid plaque pathology were transgenic mice made to over express FAD mutant APP forms. They corroborated the in vitro finding of an increase in overall APP processing and thus increase in Aβ (Games, Adams et al. 1995; Hsiao, Chapman et al. 1996), however they lacked the second hallmark of tau tangles observed in humans. Furthermore, a lack of widespread neuronal loss, raised concerns if they truly mimic AD (Irizarry, Soriano et al. 1997).

More recent triple transgenic models may prove to be a better model for AD (Oddo, Caccamo et al. 2003). These animals produced APP and tau predominantly in two brain regions that in human are most affected. The signature plaques and tangles develop progressively as the mice age. Accumulation of Aβ precedes tangles, supporting a widely held hypothesis that Aβ as the culprits in Alzheimer nerve and synaptic dysfunction. These genetically engineered mice provide a valuable tool to understand how the disease

process may begin, however in humans AD is a slow disease, one that is difficult to mimic perfectly in the short lifespan of these model animals.

APP biological & physiological function

APP isoforms are widely expressed glycoprotein in cells throughout the body. It is an integral membrane protein with a single membrane spanning domain, a large extracellular glycosylated N terminus and a shorter cytoplasmic C terminus. The most abundant form in brain (APP695) is produced mainly by neurons. It differs from longer forms of APP in that it lacks a kunitz type protease inhibitor sequence in its ectodomain(Hardy 1997; Mattson 1997). Early-onset APP mutations are clustered near the proteolytic processing sites α- ,β-, and γ-secretase cleavage sites. Production of Aβ is a normal consequence of APP proteolysis. However, only a small fraction of APP molecules undergo this fate. A majority of the mutations in APP are believed to shift a larger percentage of APP molecules being processed and thus over a lifetime increase the Aβ burden.

The normal physiological functions implicated to APP still remain diverse. Increasing evidence suggests that it has important roles in regulating neuronal survival, neurite outgrowth, synaptic plasticity and cell adhesion. Full-length APP may function as a cell surface receptor that transduces signals within the cell in response to an extracellular ligand(Cao and Sudhof 2001; Kimberly, Zheng et al. 2001). Neither a ligand nor downstream signaling cascades for APP has been clearly established. The soluble

forms of APP (sAPPα) or (sAPPβ) that are released upon ectodomain shedding may be released in response to electrical activity at synapses. For example, data supports these shed fragments regulate neuronal excitability, enhance synaptic plasticity, learning and memory through activating cell surface receptors through modulating the activity of potassium channel from activated downstream transcription factors(Scheuner, Eckman et al. 1996).

Evidence for cerebral accumulation of Aβ portion of APP and the "amyloid cascade hypothesis" as the causative events in AD is substantial. Location and number of amyloid plaques correlates closely with the particular cognitive decline and other clinical markers of the disease(Arriagada, Marzloff et al. 1992; Dickson, Crystal et al. 1995). However, total plaque burden may not correlate directly with the overall observed dementia(Terry 1996). Associated with amyloid plaques are neurfibrillary tangles (NFT) (Knowles, Gomez-Isla et al. 1998). The contributions of these tangles to overall dementia even if downstream temporally of plaque formation have proven hard to reconcile, to date. Well established is the fact that $A\beta_{42}$ is particularly prone to aggregation and may form the initial seed for subsequent plaque formation (Burdick, Soreghan et al. 1992; Jarrett, Berger et al. 1993). In vitro, incubation of intermediate pre-aggregated protofibril forms of Aβ with neuronal cell cultures results in neurotoxicity (Pike, Walencewicz et al. 1991; Lorenzo and Yankner 1994; Hartley, Walsh et al. 1999).

These initial experiments that showed synthetic fragments of Aβ can kill cultured neurons resulted in a series of studies that have revealed the chemical and cell biological

bases for the synaptic dysfunction and death of neurons in AD. Aβ pathogenesis has also been tested extensively mouse models. Transgenic mice models with increase Aβ plaque load have shown to induce neurite abnormalities and disrupt neural networks in a plaque dependent manner (Knowles, Wyart et al. 1999). Increased synaptic transmission deficits have also been observed early in development from similar transgenic mice in the absence of observable plaques, further supporting the fact that Aβ may be most toxic when it is in the form of soluble oligomers in the earliest stages of aggregation (Hsia, Masliah et al. 1999). Certain intermediated (pre-plaque) aggregates of Aβ have been shown to disrupt long-term potentiation in vivo in rats (Walsh, Klyubin et al. 2002). The notable finding that immunization of APP mutant mice with human $A\beta_{42}$ results in the removal of Aβ deposits from the brain and reversal in cognitive deficits adds to the evidence that Aβ deposition is a pivotal event in AD(Morgan, Diamond et al. 2000; Klyubin, Walsh et al. 2005).

APP proteolytic processing

How does Aβ production occur? A question of obvious intense research has resulted in a detailed understanding of the proteolytic processing of its precursor. A portion of APP molecules may be cleaved either by two ectodomain shedding proteases, α- and β-secretase. The principle cleavage is believed to be mediated by α- secretase(s) and occurs both at the cell surface and within subcellular vesicles (Fraering, Ye et al. 2005). The cleavage results in an 83-amino acid carboxy-terminal APP fragment (C83) that is retained within the membrane and a soluble N-terminal ectodomain that is released

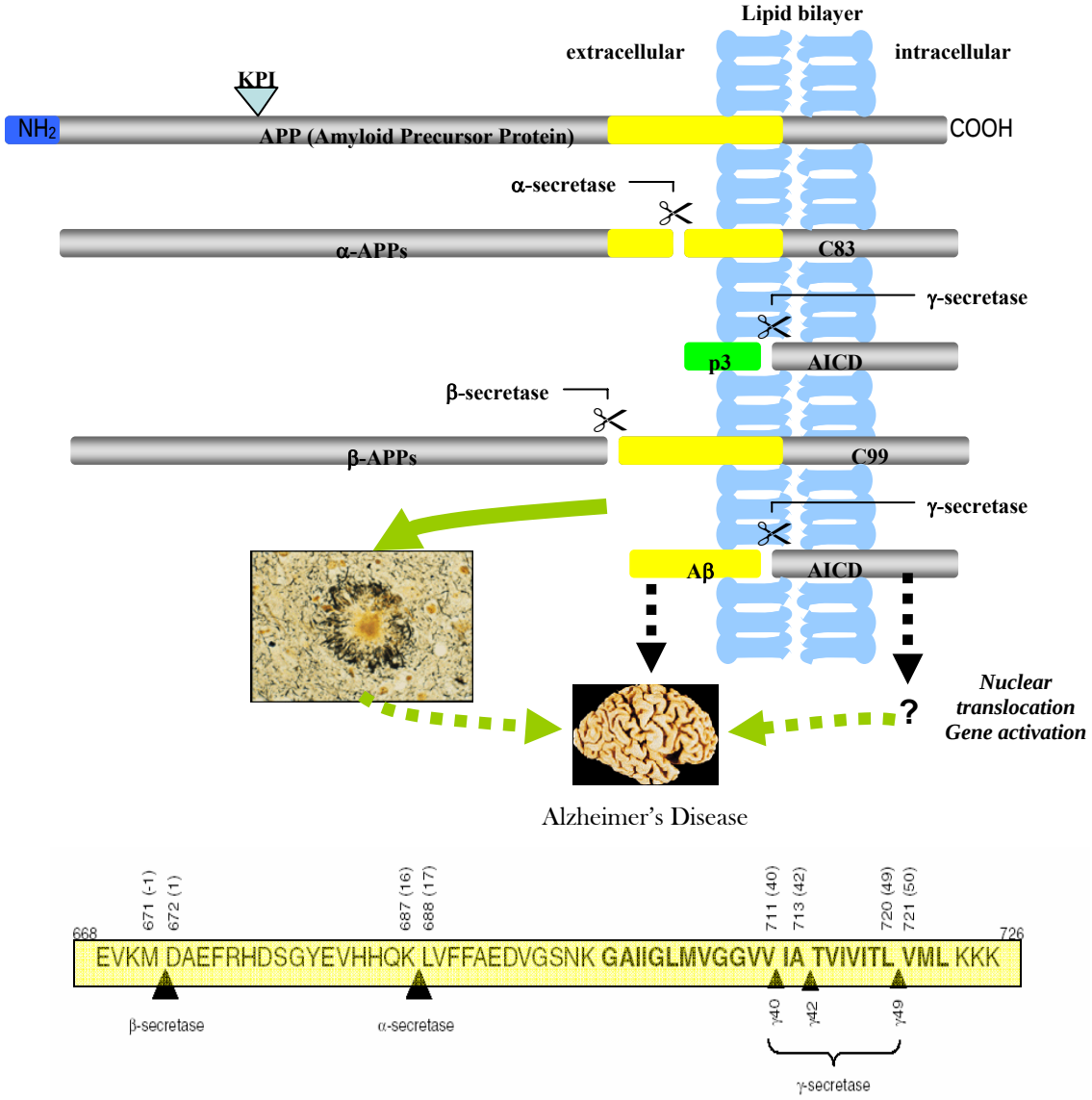


Figure 1.1. Proteolytic processing of APP. APP is a type I TM protein that possesses a signal peptide of the first seventeen amino acids on the N-terminus (dark blue). Splice isoforms expressed in the periphery contain a kunitz protease inhibitor domains (KPI). The full lenth protein is first cut by either α-secretase or β-secretase to generate α-APPs and C83 or β-APPs and C99, recpsectively. C83 and C99 serve as substrates for γ-secretase to produce p3 and Aβ, respectively. Both γ-secretase mediated cleavages release the APP intracellular domain (AICD). Also included are the amino-acid residues of Aβ and surrounding region of human APP770 showing the β-, α-, and γ- proteoytic sites. γ-Secretase cleaves at multiple sites within the transmembrane region.

from the cell surface (sAPPα). TACE (an enzyme responsible for cleavage of members of the TNF receptor family at the cell surface), ADAM9 and ADAM10 are candidates implicated in α-secretase activity (Buxbaum, Liu et al. 1998; Asai, Hattori et al. 2003). These secretases cleave APP in a sequence independent manner(Sisodia 1992). The proteolytic event occurs within Aβ fragment ~16 amino acids within the Aβ start site thus mitigating the formation of Aβ(Esch, Keim et al. 1990) (Fig 1.1).

On the other hand, amyloidogenic processing of APP involves a highly sequence specific enzyme β-Secretase (Citron, Teplow et al. 1995). The membrane-bound aspartyl protease BACE was identified simultaneously by several groups (Hussain, Powell et al. 1999; Sinha, Anderson et al. 1999; Vassar, Bennett et al. 1999; Yan, Bienkowski et al. 1999; Paganetti, Calanca et al. 2005). A second BACE gene (BACE2) was identified shortly thereafter, although the protein is not expressed in the brain and is believed to cleave APP at a site more similar to α-secretase (Farzan, Schnitzler et al. 2000; Yan, Munzner et al. 2001). Proteolytic processing of APP by BACE creates the N-terminus of Aβ, resulting in a truncated soluble ectodomain (sAPPβ) and a 99-amino acid membrane-retained CTF (C99) (Seubert, Vigopelfrey et al. 1992). These secretases may also cleave APP ~11 amino acids inside the Aβ sequence, generating a truncated Aβ peptide and an 89-amino acid CTF (Buxbaum, Thinakaran et al. 1998; Cai, Wang et al. 2001). After cleavage of APP by either α- or β-secretase, the membrane retained CTFs become immediate substrates for an unusual intramembrane proteolysis performed by the γ-secretase (Fig 1.1). The γ-secretase activity cleaves scissile bonds of the CTFs within their transmembrane regions heterogeneously. In the case of APP this would yield

species of 39 to 43 amino acids in length. Predominant Aβ species are 40 and 42 residues generated at a ratio of approximately 10:1 respectively (Fraering, Ye et al. 2005). Initial deposited species in diffuse plaques may consist of the $A\beta_{42}$ variety (Iwatsubo, Odaka et al. 1994). Aβ strongly predisposes itself to aggregation with the addition of the two hydrophobic residues at the C-terminus (Harper, Lieber et al. 1997; Harper, Wong et al. 1997; Walsh, Lomakin et al. 1997).

Presenilin Genetics

Two additional genes responsible for familial autosomal dominant AD have been identified in the human genome: presenilin 1 (PS1) and presenilin 2 (PS2). Mutations in these as well as APP account for up to 50% of familial cases of early-onset AD (Haass 1997; Hutton and Hardy 1997). To date, 20 mutations in the APP gene, 124 mutations in the PS1 gene and 8 mutations in the PS2 gene have been described worldwide (Cruts, van Duijn et al. 1998; Campion, Dumanchin et al. 1999). The discovery of PS genes as the major pathogenic genes for early onset FAD, prompted the identification of the γ-secretase. Evidence for PS as the cofactors that provide the catalytic activity of the γ-secretase has accumulated significantly over the years. First, FAD-linked mutations of PS shift the preferred site of γ-cleavage from position 40 to 42 of Aβ (Thinakaran, Borchelt et al. 1996). Second, γ-secretase activity is diminished in cells derived from PS knockout mice (De Strooper, Saftig et al. 1998). Third, mutations in either of the two aspartate residues buried within the 6^{th} and 7^{th} transmembrane domains of PS abolishes γ-secretase activity (Wolfe, Xia et al. 1999).

Endogenously present in every type of mammalian cell, PS1 and PS2 are highly homologous 8-TM proteins. They undergo endoproteolysis, and the resulting amino-terminal (NTF) and carboxy-terminal fragments (CTF) remain closely associated (Capell, Grunberg et al. 1998; Strooper and Annaert 2001). The latter forms of PS are the predominant forms found in mammals (Thinakaran, Borchelt et al. 1996). Over expression of PS has little effect on the stability or levels of the fragment (Thinakaran, Harris et al. 1997). The over expressed full length molecules of PS are rapidly degraded through ubiquitination and proteasome-mediated degradation (Kim, Pettingell et al. 1997; Steiner, Capell et al. 1998). Additional mechanisms such as phosphorylation or caspases may regulate presenilin mediated turnover, however the mechanisms behind these events remain to be fully elucidated (Kim, Pettingell et al. 1997; Grunberg, Walter et al. 1998).

To gain further insights into the role PS may play in APP metabolism, several biochemical and subcellular localization studies have been performed. APP co-immunoprecipitates with PS1 and PS2 and these interactions occur predominantly in the endoplasmic reticulum (ER) but also the Golgi (Kovacs, Fausett et al. 1996; Cook, Forman et al. 1997; Hartmann, Bieger et al. 1997; Xia, Zhang et al. 1998). Furthermore, direct substrates of γ-secretase such as APP C99/C83 fragments, can co-immunoprecipitate with PS (Verdile, Martins et al. 2000; Xia, Ray et al. 2000). Cell membrane preparations on gradients demonstrated that FL-PS is present in the ER while the NTF and CTF are present primarily in the Golgi (Xia, Zhang et al. 1998; Zhang, Kang et al. 1998; Annaert, Levesque et al. 1999). Paradoxically, a majority of Aβ has

been shown to be generated during the endosomal recycling of APP from the cell surface (Fraering, Ye et al. 2005), making it difficult to reconcile how presenilins would exert their effects on Aβ generation if there is spatial dislocalization between enzymatic activity and substrate (Annaert and De Strooper 1999). Recently, careful localization coupled with the identification of additional proteins critical for the assembly and overall trafficking of a γ-secretase complex has revealed that the catalytic active presenilins may not be limited to the ER and Golgi. In fact, presenilins and their homologs have been localized to the cell surface in Drosophila embryos (Fraering, Ye et al. 2005), in transfected cells (Takashima, Sato et al. 1996), and at the endogenous cellular levels (Schwarzman, Singh et al. 1999). Furthermore, PS immunoreactivity has been identified using subcellular fractionated vesicles just apposed to the plasma membrane (Efthimiopoulos, Floor et al. 1998) and using electron microscopy on primate brains (Lah, Heilman et al. 1997). Therefore, PS is likely to be found where ever Aβ generation takes place.

Presenilin Function

Notch is a single pass TM receptor. It was originally discovered in the fruit fly *Drosophilar*, where partial loss-of-function caused a notched wing. These mutants also generated a neurogenic phenotype where excess neural precursors were formed at the expense of epidermal ones (Artavanis-Tsakonas, Rand et al. 1999). Initial clues to PS function came from the identification of the C. elegans PS sel-12 in a screen for mutants that impact Notch function (Levitan and Greenwald 1995). Impairment of Notch

functions in the SEL-12 mutant's result in a partial loss-of-function egg-laying defective (Egl) phenotype. Expression of human PS1 or PS2 driven off the sel-12 promoter rescued the Egl defects. Thus, PS are required for Notch function and human PS can substitute for C. elegans PS, indicating conserved function in Notch processing from humans to C.elegans (Levitan, Doyle et al. 1996). PS1 knockout mice also have defects that are reminiscent of Notch knockout phenotypes and re-introduction of wtPS1 or FAD mutant PS1 rescues the lethality of the PS1 knockout mouse. Cell cultures from these mice express very little NICD and show a reduction in Notch signaling, and transfection of wt PS1 or FAD mutant PS1 rescued Notch cleavage and nuclear translocation. The residual NICD present may be produced by endogenous PS2.

The PS1/2 KO mouse displays a number of severe phenotypes that are similar to the Notch1 knockout mouse (Shen, Bronson et al. 1997; De Strooper, Annaert et al. 1999). However, the mice express additional phenotypes that may be indicative of absence of signaling by all four Notch homologues or loss of PS cleavage of other substrates(Wong, Zheng et al. 1997). Transfection studies in cell lines derived from a PS1/2KO mouse confirm that loss of both PS results in the complete absence of NICD production (Herreman, Serneels et al. 2000). The severity of the PS1/2KO phenotype versus the single knockouts demonstrates that PS1 and PS2 have overlapping functions and that while PS1 can compensate for loss of PS2, PS2 is unable to compensate for loss of PS1(Donoviel, Hadjantonakis et al. 1999; Herreman, Hartmann et al. 1999). These data confirm the dependence on PS for γ-secretase cleavage of Notch.

The Notch protein functions as a receptor at the cell surface and mediates cell-cell signaling interactions to specify cell fates within an equivalence group, a role that is particularly important during development. Notch is activated by a proteolytic cascade similar to that of APP. During transport through the secretory pathway, Notch is constitutively cleaved by furin in the trans-Golgi network at cleavage site 1 (S1) to form transmembrane-intracellular Notch (Logeat, Bessia et al. 1998; Rand, Grimm et al. 2000). Upon interaction with ligand, furin-cleaved Notch undergoes a second proteolytic cleavage at S2 (site 2). The ectodomain bound ligand is endocytosed into the ligand-expressing cell (Parks, Klueg et al. 2000), while the Notch extracellular truncation (NEXT) fragment remains embedded in the membrane (Mumm, Schroeter et al. 2000). NEXT serves as the substrate for a third proteolytic cleavage at S3 (site 3) that releases the Notch intracellular domain (NICD) fragment from the membrane, allowing it to translocate to the nucleus and interact with transcription factors (Jarriault, Brou et al. 1995; Schroeter, Kisslinger et al. 1998). NEXT may also be cleaved at S4 (site 4) in the middle of the transmembrane domain to form the Nβ peptide that is similar to the p3 and Aβ peptides produced from cleavage of APP C83 and C99(Okochi, Steiner et al. 2002; Zhang, Ye et al. 2002).

Release of NICD is performed by the same presenilin-dependent γ-secretase activity that cleaves APP to release CTFγ. APP and Notch were the first recognized substrates of a novel mechanism to activate receptors for signaling. The first step in regulated intramembranous proteolysis (RIP) is cleavage by a ectodomain shedase that results in removal of the large ectodomain (Brown, Ye et al. 2000). The remaining

membrane-embedded CTF containing an extracellular domain with less then 30 amino acids is then a substrate for proteolysis within the transmembrane domain by presenilin and cofactor protein complex (Wolfe, Xia et al. 1999). Processing and release of AICD or NICD is believed to modulate transcription downstream.

Although in vivo data strongly supports an essential role for PS in γ-secretase activity little is known about the structure of γ-secretase, the mechanism it utilizes for proteolysis, or the regulation of cleavage. Charged residues are rare within TM domains. However, PS have two potentially charged aspartic acid residues in TM6 and TM7 that are completely conserved in all PS. Mutations of either or both of these two residues in PS1 (D257A and D385A) abrogate presenilinase processing within the TM6 and TM7 loop (Wolfe, Xia et al. 1999). Treatment of cells with diflouroketone and aldehyde peptidomimetic inhibitors designed to mimic the Aβ42 production at low concentrations, and at high concentrations, block production of Aβ40 and Aβ42 as well as NICD production and nuclear translocation (De Strooper, Saftig et al. 1998; Wolfe, Citron et al. 1998). The aspartyl protease inhibitor Pepstatin A inhibited production of Aβ species equivalently. Studies with such compounds suggested that the γ-secretase enzyme they block is an aspartyl protease. They further hypothesized loose sequence specificity and an α-helical model for the γ-secretase cleavage site in efforts to explain the effects of FAD APP mutations and correlate the position of the heterogeneous cleavage sites within the transmembrane domain of APP (Wolfe, Xia et al. 1999; De Jonghe, Esselens et al. 2001).

Biochemical characterization of γ-secretase in a membrane-based assay using the APP C99 substrate demonstrated that Aβ40 and Aβ42 production have similar pH requirements, and the aspartyl protease inhibitor Pepstatin A inhibited production of both equivalently (Zhang, Song et al. 2001). When inhibitor profiles of seven γ-secretase inhibitors representing a range of structures were compared for APP and Notch in parallel and at numerous concentrations, the rank order of potency was equivalent, implicating a single proteolytic activity in the cleavage of both substrates (Schroeter, Kisslinger et al. 1998). The demonstration of competition between APP and Notch for γ-secretase further supports the proposal that a single γ-secretase activity cleaves both substrates (Lleo, Berezovska et al. 2003). Thus γ-secretase appears to be an aspartyl protease that cleaves both APP and Notch.

A growing list of putative γ-secretase substrates

Numerous substrates have now been identified for the γ-secretase intramembrane-cleaving activity. In addition to APP and Notch and their respective isoforms, γ-secretase also cleaves:

Epithelial cadherin (E-cadherin): a protein that functions in calcium-dependent cell-cell adhesion. Proteolysis serves to dissociate E-cadherin-catenin complexes and release β-catenin into the cytoplasm. Release of the cytoplasmic E-cadherin to the cytosol increases the levels of soluble beta- and alpha-catenins. Thus, γ-secretase stimulates disassembly of the E-cadherin- catenin complex and increases the cytosolic pool of beta-catenin, a key regulator of the Wnt signaling pathway. (Marambaud, Shioi et al. 2002).

Neuronal Cadherin (N-cadherin): mediates calcium-dependent cell-cell adhesion through homophilic interactions that are important in neuronal and synaptic functions. γ-Secretase protease activity promotes cleavage of N-cadherin to produce its intracellular domain peptide, N-Cad/CTF2. The intracellular domain is believed to bind the transcription factor CBP and promote its proteasomal degradation, inhibiting CRE-dependent transactivation. Thus, the γ-secretase product N-Cad/CTF2 functions as a potent repressor of CBP/CREB-mediated transcription (Marambaud, Wen et al. 2003).

Nectin-1: a protein that is a member of the immunoglobulin superfamily. It mediates Ca2+ independent cell-cell adhesion and also releases β-catenin into the cytoplasm. However, Nectin-1 cleavage was only observed upon (TPA) treatment of cell cultured cells and conditions under which endogenous cleavage may be observed remain elusive. Similar to nectin-1, other members of the immunoglobulin superfamily involved in synapse formation may also serve as substrates for γ-secretase-like intramembrane proteolytic activity (Kim, Ingano et al. 2002).

Low density lipoprotein receptor-related protein (LRP): is cleaved first by furin, then by a metalloprotease and finally, by γ-secretase to release an LRPICD that may modulate transcription. LRPICD may be translocated to the nucleus, where it colocalizes in the nucleus with a transcription modulator, Tip60, which is known to interact with Fe65 and with the APP-derived intracellular domain. The intracellular domain of LRP may have a novel signaling function, negatively impacting transcriptional activity of the APP, Fe65, and Tip60 complex in the nucleus (May, Reddy et al. 2002).

CD44: is a major adhesion molecule for the extracellular matrix components and is implicated in a wide variety of physiological and pathological processes. The

intramembranous cleavage of CD44 induced by (TPA) treatment or mechanical scraping is blocked by gamma-secretase inhibitors or in PS-deficient mouse embryonic fibroblasts. In the future, understanding CD44-dependent signal transduction and a potential role of the γ-secretase activity in the functional regulation of adhesion molecules may elucidate aspects of tumor cell growth and metastasis (Lammich, Okochi et al. 2002).

ErbB-4: a receptor for epidermal growth factor (EGF). ErbB-4 is a receptor tyrosine kinase that functions in cell proliferation and differentiation. In response to binding of its ligand heregulin (HRG) or activation of protein kinase C (PKC) by (TPA), the ErbB-4 ectodomain is cleaved by a metalloprotease. ErbB-4 intracellular domain is generated by the γ-secretase from the membrane and facilitates its translocation to the nucleus. γ-Secretase cleavage of ErbB-4 is believed to represent another mechanism for receptor tyrosine kinase-mediated signaling (Ni, Murphy et al. 2001).

Notch ligand Delta1 & Jagged2: may play dual roles as activators of Notch receptor signaling and as receptors that mediate nuclear signaling events via γ-secretase generated cytoplasmic domains (Ikeuchi and Sisodia 2003).

DCC (deleted in colorectal cancer): a substrate for metalloprotease-dependent proteolytic processes that results in shedding of its ectodomain. Putatively important in receptor-mediated intracellular signaling pathways that are critical in regulating glutmatergic synaptic transmission and memory processes (Taniguchi, Kim et al. 2003).

Syndecan3: The heparin sulfate-bearing syndecan ectodomain undergoes ligand-activated or stress induced proteolytic cleavage and shedding. The released intracellular domain negatively regulates the plasma membrane targeting of the transcriptional cofactor CASK (Schulz, Annaert et al. 2003).

P75 NTR: a neurotrophin receptor implicated in diverse functions that include promoting survival, inducing apoptosis, enhancing neurite growth, facilitating growth cone collapse, and mediating differentiation and enhancing proliferation. MAG binding to cerebellar neurons induces α- and then γ-secretase proteolytic cleavage of p75, in a protein kinase C-dependent manner, and that this cleavage is necessary for both activation of Rho and inhibition of neurite outgrowth (Domeniconi, Zampieri et al. 2005).

L1, (the neural cell adhesion molecule L1): is involved in the development of the nervous system promoting neuronal migration, neuronal survival, neurite outgrowth, and myelination, as well as axon guidance, fasciculation, and regeneration. ADAM10 and ADAM17 are involved in the release of the extracellular domain of L1. C-terminal fragment is further processed through γ-secretase activity, suggesting the possibility that L1 may also contribute to intracellular signal transduction pathways (Maretzky, Schulte et al. 2005).

The site of proteolysis slightly varies among the different substrates. It is mostly located close to the membrane-cytosol interface. The consensus sequence for PS-dependent γ-secretase recognition/cleavage is very relaxed or does not exist at all, although a Val residue right after the cleavage site is present in most cases. Interestingly, based on the sequence of the γ-secretase generated AICD, a new intramembrane cleavage site of APP (ε-cleavage) has been identified (Weidemann, Eggert et al. 2002). APP ε-cleavage is also PS-dependent and the ε-site is located near the membrane-cytosol interface in an equivalent position as the Notch S3 site and other reported cleavage sites. The lack of dependence for catalytic activity on the precise sequence surrounding the

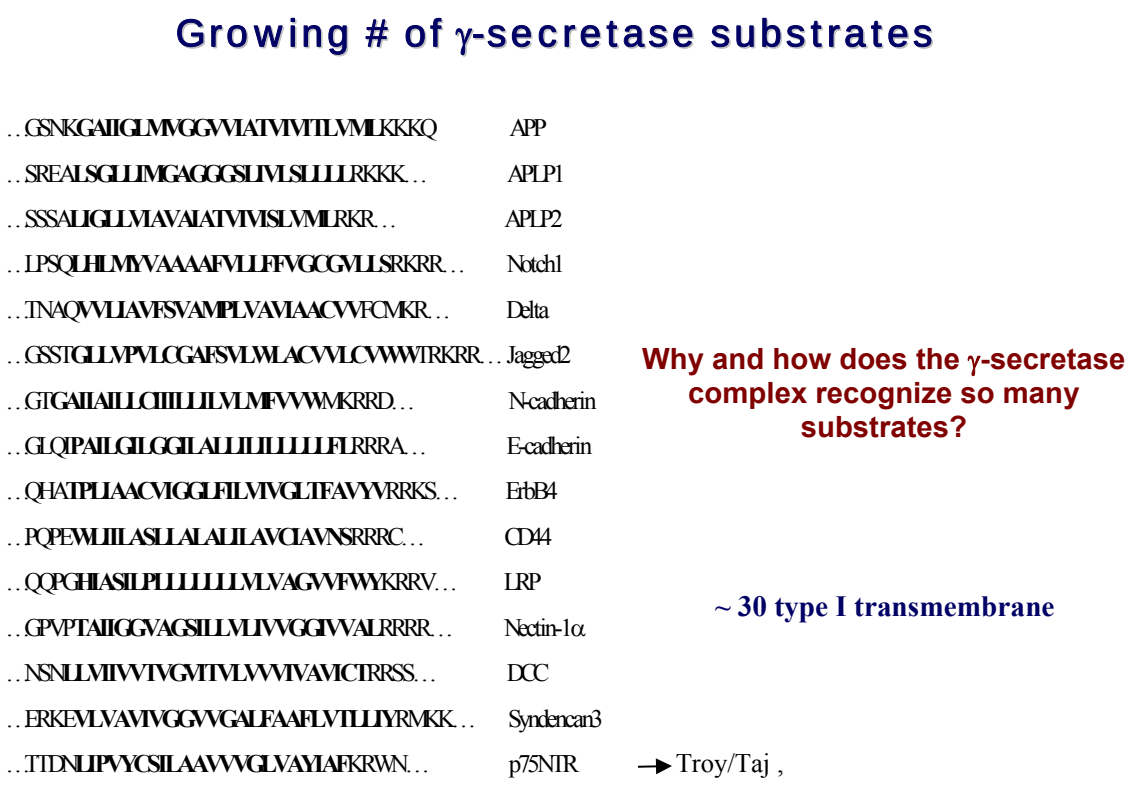


Figure 1.2. A growing list of γ-secretase substrates. In addition to Notch and APP and their homologs a growing list of gamma secretase substrates have been identified. A question that has puzzled us and others in the RIP field is how does the gamma secretase complex recognizes so many different substrates. There are no common sequence motifs within the transmembrane regions (bold) of these substrates. What is common is their polarity. They are all group one transmemebrane proteins. They all also are processed by the prerequisite ectodomain shedding that leaves less then 50 amino acid stub extracellulary before being cleaved intramembranously by gamma secretase.

cleavage site may suggest that the regulation of cleavage specificity is achieved indirectly. Alternatively, others have hypothesized multiple cleavage events that progress sequentially or simultaneously from the membrane-cytosol interface towards the mid-portion of the lipid bi-layer (Zhao, Mao et al. 2004).

Recent developments reveal a large number of putative type-I membrane proteins that may participate in cell signaling through γ-secretase dependent RIP events (Fig 1.2). γ-Secretase appears to have very broad substrate specificity. The only known prerequisites for γ-secretase substrates are (1) type I membrane proteins and (2) shedding the bulk of the extracellular domains from the full-length precursor proteins. Thus, one critical unanswered question concerning γ-secretase mediated RIP is how the γ-secretase complex recognizes its many heterogeneous substrates which seem to have no specific amino acid sequences or substrate recognition domains common amongst them. One factor important in initial substrate recognition may be the orientation as well as origination of short extracellular domain after the primary processing event.

The γ-secretase complex

Given the growing body of evidence that γ-secretase is essential for the proteolysis of many substrates with important roles in normal cellular physiology and pathophysiology, attention over the last few years has focused on identifying the

components of the macromolecular γ-secretase complex, its regulated assembly, and its biochemical activities. Evidence that PS might be a novel type of aspartic protease was

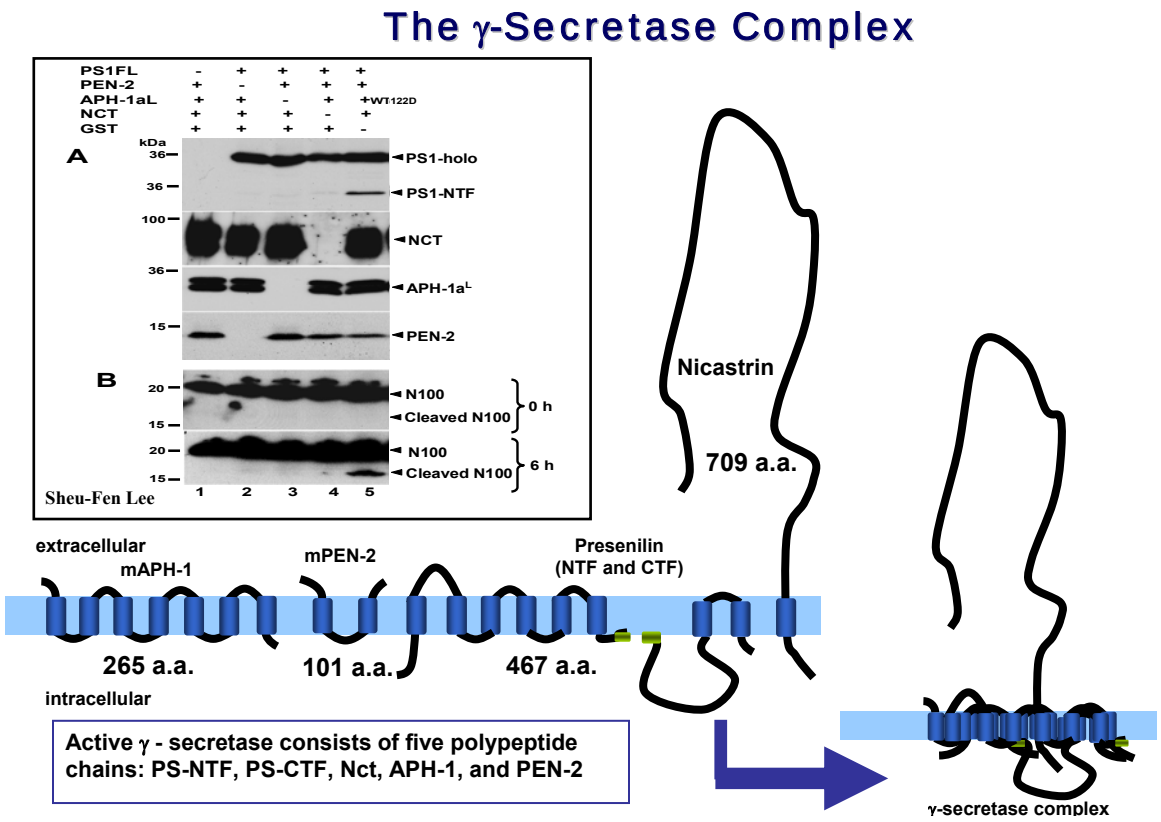


Figure 1.3. Topology of γ-secretase complex members. PS1 and PS2 are predicted to have 8 TM domains. The N- and C-terminal tails and the loop are oriented towards the cytoplasm. The TM aspartates are embedded within TM domains 6 and 7. The site of endoproteolysis is located at the methionine in the hydrophobic stretch of amino acids between TM domains 6 and 7. Genetic screens in C. elegans that cause notch phenotype similar to loss of presenilin or nicastrin identified Aph1 (seven TMs) and Pen2 (two TMs). Aph-1, Pen-2 and Presenilin are polytopic membrane ptoeins. Nicastrin is the odd one out of the four in that it only contains a single transmembrane span and has a very large ectodomain. If we were to consdier equal molar ratios of each component for an active enzyme Nicastrin would make up almost half of the complex. All four components are required for γ-secretase acitivity as shown form reconstitution experiments performed in SF9 cells where no endogenous components or activity is observed.

gleaned when transition-state analog inhibitors for γ-secretase, which were predicted to complex with the catalytic domain of the enzyme, in membrane fractions solubalized in CHAPSO *in vitro* found these inhibitors directly bound to the amino- and carboxy-terminal fragments of PS (Wolfe, Citron et al. 1998). These results strongly supported the notion that PS, especially in its mature endoproteolyzed form, constitutes the catalytic subunit of γ-secretase. However, biochemical studies indicated that γ-secretase activity was associated with a highly stable, high-molecular weight protein complex of ~250-1000kDA in size (Yu, Chen et al. 1998). These findings spurred on the search for binding partners (cofactors) of PS that stabilized and activated the γ-secretase complex (Fig 1.3).

The first protein cofactor of PS to be identified through co-immunoprecipitation studies in HEK cells. Antibodies to PS pulled down Nicastrin (NCT), a single-pass TM protein that is evolutionarily conserved from C.elegans and Drosophila through mammals. NCT was observed in high molecular weight fractions, which included PS1 and PS2, and co-localized with ER and Golgi markers. RNA interference (RNAi) knockdown of aph-2 (a NCT homolog) in C. elegans resulted in hypohlasia of the anterior pharynx, reminiscent of that seen in the glp-1 (a notch homolog) mutant (Yu, Nishimura et al. 2000). Ablation of NCT in Drosophila led to embryonic lethality, which could be rescued by overexpression of NICD (Hu, Ye et al. 2002). Taking these results into account, NCT seemed to be essential for notch signaling through cleavage and

activation of NICD. However, overexpression of NCT alone in mammalian cells did not increase the levels of the functional γ-secretase complex.

A screen against a sel-12 partial loss of function mutation in C. elegans sought to identify PS interactors in the glp-1/Notch pathway (Francis, McGrath et al. 2002). It identified two additional multi-TM, ER/Golgi resident proteins, APH-1 and a protein designated PEN-2 (presenilin enhancer). APH-1 is a seven-TM protein with the C-terminus located in the cytoplasm, while PEN-2 has two TMs with the N- and C-termini located in the lumen/extracellularly. Both APH-1 and PEN-2 interacted genetically with sel-12, hop-1 and aph-2/NCT, and loss-of-function alleles displayed phenotypes similar to those of sel-12 and glp-1.

Current evidence supports presenilin and its endoproteolytic fragments as the catatlytic subunit of the γ-secretase complex. However, the precise function for Aph-1, Pen-2, and nicastrin in the complex and in intramembrane proteolysis remains unclear. Evidence for Aph-1 and Pen-2, points to a role in the assembly and maturation process of the complex. Unlike the polytopic membrane proteins presenilin, Aph-1 and Pen-2, nicastrin is a type-1 membrane glycoprotein with a large and unique ectodomain. In addition to evidence for nicastrin ectodomain playing a role in structural assembly, maturation, and activity of γ-secretase; preliminary data also suggested a putative role for the nicastrin ectodomain functioning as a receptor for γ-substrates.

1) Nicastrin can bind to the immediate substrates of (C100 & N99) gamma secretase under conditions that disrupt the core complex.
2) A portion of nicastrin ectodomain has sequence similarity to mono-zinc aminopeptidases; however no catalytic activity has been detected to date. Alterations, in these conserved regions of nicastrin ectodomain have effects on intramembrane cleavage of γ-substrates, particularly APP.
3) Studies using inhibitor affinity chromatography of the complex co-purifies the γ-substrates and points to a yet to be identified pre-docking site distinct form the active site of the complex.

The central goal of this thesis is to identify the role nicastrin in γ-secretase. We hypothesized that nicastrin, the only component of the core complex which contains a large extracellular domain, is a protein that may serve as a receptor for substrates. Specifically, I show that show that the APP or Notch derived γ-secretase substrates (C100 and N99) stoichiometrically, directly, and functionally interact with nicastrin. This interaction is mediated by the extracellular N-terminal stub of the substrate and the ectodomain of nicastrin. We find that a conserved glutamate (Glu333) within the extracellular aminopeptidase domain of nicastrin to be essential for substrate docking and γ-secretase catalytic activity. Chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduce the binding and cleavage of substrate by γ-secretase. Taken together these results define an interaction site between the extracellular domains of both the substrate and nicastrin and suggest a putative function for nicastrin as a receptor for γ-secretase substrates. The γ-secretase complex has been traditionally viewed as a hub for signal transduction of substrates such as Notch and APP. The mechanism by which a

broad range of substrates may be recognized and subsequently cleaved, as demonstrated in this thesis, supports a mutually inclusive function as a protease that has evolved to simply dispose transmembrane domains thus controlling the repertoire of a class of proteins present in the membrane.

Chapter 2

Aph1 is a required cofactors of the γ-secretase complex for intramembrane proteolysis.

Attributions: In this chapter, I made significant contributions in the initial cloning and characterization of APH-1 and to a lesser extent PEN-2. Some experiments were performed collaboratively and others where my involvement was purely indirect. It is difficult and may be inaccurate to attribute each aspect of the work individually except to acknowledge all authors in the publications below and particularly Sheu-Fen Lee and Hongqia Li whom I worked very closely with during the earlier years of my graduate studentship.

Publications: Data in this chapter is published in Lee *et al.,* Journal of Biological Chemistry 2002 and Luo *et al.* Journal of Biolocial Chemistry 2003.

Abstract

A central hallmark in the disease pathogenesis associated with Alzheimer's disease (AD) is the accumulation of the amyloid β-protein (Aβ). The γ-secretase performs the final step in the generation of Aβ from amyloid precursor protein (APP). Presenilin and nicastrin were essential components that form a complex critical for this unusual activity. However, the true identity of the aspartyl protease that cleaves within the transmembrane remained an area of intense investigation. Purification of cell fractions that maintained such enzymatic activity migrated in the molecular weight range larger then a simple stoichiometric relationship between presenilin and nicastrin. The enzymatically active protease consisted of presenilins that are endoproteolytically cleaved into N-terminal (NTF) and C-terminal (CTF) fragments. Overexpression, of presenilin results in an accumulation of full length uncleaved forms. Similarly, a highly glycosylated form of nicastrin (mature nicastrin) was associated with enzymatically active enzyme. Overexpression, of nicastrin resulted in an accumulation of an immature underglycosylated form of nicastrin. This generated the 'limiting factor' hypothesis, that unknown cofactors were required to form an active core complex. In this chapter, I present our efforts to identify and characterize a multipass membrane proteins mammalian APH-1 (mAPH-1) as a functional component of the γ-secretase complex. Upon cloning the mammalian forms, this membrane protein was shown to associate with nicastrin and presenilin in co-immunoprecipitations. Specific knockdown approaches of

endogenous forms of this protein resulted in a decrease of active complex and reduction in γ-secretase products.

Introduction

Inactivation of presenilins in *D. melanogaster*, *C. elegans*, and *M. musculus* results in the disruption of the intramembrane proteolysis of Notch and APP (De Strooper, Saftig et al. 1998). Substantial evidence accumulated that presenilins appeared to contain the critical intramembranous di-aspartates for this enzymatic activity. Despite this evidence it remained clear that presenilins were alone, insufficient to perform cleavage of cell surface receptors and the true identity of these atypical aspartyl protease remained to be discovered.

In a highly regulated fashion, presenilins are endoproteolytically cleaved into a 30-kDa N-terminal (NTF) and a 20-kDa C-terminal fragment shortly after they are biosynthesized (Thinakaran, Harris et al. 1997). Remaining full length presenilin is rapidly degraded by the proteasome and is difficult to detect at endogenous levels (Kim, Pettingell et al. 1997). The NTF and CTF fragments are stable and incorporated in a 1:1 stoichiometry in protein complexes migrating at apparent molecular weights of ~150 to 1000 kDa using methods of glycerol gradients, gel filtration or native gels (Capell, Grunberg et al. 1998; Kimberly, LaVoie et al. 2003). The biologically active forms of PS reside in these complexes. Upon overexpressing presenilin only uncleaved precursor protein is detected, which is not incorporated into the complexes. Stable expression of PS

variants results in replacement of endogenous PS over time, however no increase in γ-secretase activity is observed (Thinakaran, Harris et al. 1997). This led to the 'limiting factor' hypothesis and that unknown cofactors were required to fully form an active core complex.

Analogously, nicastrin that is highly N-linked glycosylated is selectively present with the active core complex. Overexpression of nicastrin does not generate more mature protein, similar to the strict regulation of the formation of presenilin heterodimers (Yu, Nishimura et al. 2000). Although a portion of stably expressing nicastrin variant could replace endogenous nicastrin, increase in formation of presenilin heterodimers or amount of active core complex unaffected. Absence of presenilin severely limits the maturation of nicastrin, while absence of nicastrin severely limited presenilin hetrodimer formation. Overexpression of both proteins does not increase their respective mature types. In sum, these findings demonstrated that the active core γ-secretase complex required presenilin and nicastrin, presenilin was tightly regulated by endoproteolysis, nicastrin incorporation was regulated via glycosylation and additional limiting cofactors facilitated or regulated such processes.

Aided by the fact that γ-secretase activity resided in a high-molecular-weight complex and that genetic screens for enhancers identified additional PS-dependent Notch-deficient phenotype in *C. elegan* we attempted to clone and characterize the unknown mammalian cofactors. We established extensive bioinformatics approaches to identify these factors. Biochemical co-immunoprecipitations and purification schemes

were used to establish physical association. Loss of these endogenous cofactors resulted in as similar loss of presenilin and nicastrin as well as inactivation of the intramembrane proteolysis of Notch and APP.

Methods

cDNA constructs - Full-length mAPH-1 cDNAs were amplified by PCR form either the human glioblastoma U118-MG cDNA library or the I.M.A.G.E. clones (numbers 3457593, 4177580, and 264868). The U118-MC cDNA library was obtained from D. Young. Expression constructs were generated by inserting the mAPH-1 cDNAs in frame with the Myc and His epitope of pcDNA4 (Invitorgen) at the carboxyl terminus of mAPH-1. Full length human PEN-2 was amplified by PCR form I.M.A.G.E. clone (3901082). Expression construct was generated by inserting full length cDNA in frame with HA in pRK5 or pCMV at the amino terminus of PEN-2. Chromosomal localizations and genetic map positions were obtained from public genetic and transcriptional maps (www.ncbi.nlm.nih.gov). Sequence alignment was performed by using the Multalin program (prodes.toulouse.inra.fr/multalin).

Cell Lines and Transfection – HEK293 cells stably or HeLa cells transiently trnasfected with the Myc-His-tagged mAPH-1 were used to evaluate the interactions of mAPH-1 with presenilin and nicastrin. HeLa cells were stably transfected with a pcDNA3.1/APP Swedish construct to generate the HeLa/APP Swedish stable cell line used for detecting APP processing in the small interfering RNA (siRNA) studies. Native HeLa cells were also used in the siRNA experiments. Transient transfections were performed using FuGENE-6 transfection reagent (Roche Diagnostics). Coexpression of multiple cDNA

constructs was achieved by cotransfection of equal amounts of DNA, resulting in comparable levels of protein expression.

RNA Interference – Small interfering RNA oligonucleotides (University of Texas Southwestern RNA Synthesis Core) directed against:

mAPH-1a :

(5'-GAAGGCAGATGAGGGGTTATT-3' and 5'-GATCACCCATCTCCATCCGTT-3')

mAPH-1b:

(5'-TACCCTATCTGACTCCTTGTT-3' and 5'CAAGATGGACCAACACAGTT-3'),

nicastrin: (5'-GGGCAAGTTTCCCGTGCAGTT-3')

Presenilin-1: (5'-GGTCCACTTCGTATGCTGGTT-3')

PEN-2: (5'-AAGGCTATGTTTGGCGCTCAG-3')

Control siRNA: (5'-AAATGTGTGTACGTCTCCTCC-3') was designed by random nucleotides selection without targeting any sequence in the genome. Oligos were transfected into native HeLa cells or HeLa/APP Swedish stable cell lines according to the guidelines recommended. mAPH1-a siRNA oligonucleotides used in the study target both mAPH-1aL and mAPH-1aS.

Antibodies – H2B (H2B-1 and H2B-2), H2D (H2D-1 and H2D-2), and H2E (H2E-1 and H2E-2) were raised in rabbits against hydrophobic mAPH-1a peptides specifically recognizing mAPH-1aL in co-immunoprecipitiation and Western blotting experiments. H2D was further affinity-purified and used to demonstrate the interaction of endogenous mAPH-1aL with endogenous presenilin and nicastrin in brain tissues. Myc-His tagged mAPH-1 proteins were visualized using the monoclonal anti-Myc antibody 9E10 (ATCC) and immunoprecipitated with rabbit polyclonal anti-c-Myc antibody A-14 (Santa

Cruz Biotechnology). Other antibodies include rabbit polyclonal and mouse monoclonal anti bodies against presenilin-1 and presenilin-2 amino and carboxyl-terminal fragments (Chemicon and Oncogene, respectively), rabbit polyclonal anti-nicastrin antibodies (ABR and Calbiochem, respectively) antibodies against full length APP and COOH-terminal α- and β-secretase derivatives (Sigma and Chemicon, respectively) and anti-α-tubulin (Sigma). Rabbit polyclonal antibody PNT2 was generated (in Dr. Thinakaran's laboratory at the University of Chicago) against the NH2-terminal 26 amino acids of PEN-2 and polyclonal antibody CR8 was generated (in Dr. Kim's laboratory at Columbia University) against the COOH-terminal 25 amino acids of PEN-2.

Biochemical Methods, APP and Notch Cleavage Assays – Co-immunoprecipitation and Western blotting were performed as described in Appendix. Nickel-nitrilotriacetic acid (Ni-NTA) agarose affinity pull-down experiments were performed according to the manufacturer's instruction (Qiagen). For substrate assays native HeLa cells were transfected with appropriate siRNAs or control RNA oligonucleotides for 56-96 h to inactivate target genes. The resultant cells ere subsequently transfected with proper reporter constructs analyzed for APP and Notch cleavage in either the luciferase reporter gene assays or the NΔE immunoblotting analysis. Luciferase reporter systems were provided by T. Sudhof and J. Lundkvist. NΔE and NICD constructs were from R. Kopan. For Aβ analysis, HeLa/APP Swedish stable cell lines were treated with the appropriate siRNAs for 56-96h and then changed to fresh media for 16h. Aβ was immunoprecipitated form the conditioned media by polyclonal antibody, separated on Bicine/Tris SDS gel and immunoblotted with Aβ monoclonal antibody.

Results

C. elgans genetics revealed that aph-1 interacts with sel-12 and hop-1 and may be an enhancer of γ-secretase activity (Francis, McGrath et al. 2002; Goutte, Tsunozaki et al. 2002). Genes essential of notch/glp-1 signaling now include the presenilin homologs, sel-12 and hop-1, along with aph-2 (the nicastrin homolog), in addition to these newly identified genes aph-1 and pen-2. Aph-1 was shown to be necessary in the cell surface localization of APH-2 (Nicastrin) in C. elegans as well as in Drosophila cells. It affects presenilin levels as well as the cleavage of substrates APP and Notch.

Here we initiated the cloning and characterization of human APH-1. Bioinformatics and cloning from brain libraries or IMAGE clones allowed for the isolation of two mAPH-1 (mAPH-1a and mAPH-1b). These genes have a high homology to worm aph-1 with several conserved glycine residues conserved throughout species. Two splice variants exist for mAPH-1a. (1) mAPH-1aL encodes a longer open reading frame of 265 residues; (2) mAPH-1aS encodes a shorter open reading frame of 247 residues. mAPH-1b encodes a 257 amino acid protein (Fig 2.1). Carboxy-terminal Myc and His epitopes were tagged to these three versions of mAPH-1. Hek293 cells that are transiently or stably transfected with these constructs reveal a band of 30KDa which matches the predicted molecular weight for these proteins.

Antibodies against mAPH-1a were raised in rabbits, to detect endogenous mAPH-1. We found that mAPH-1 interacts with nicastrin and the presenilin heterodimers in

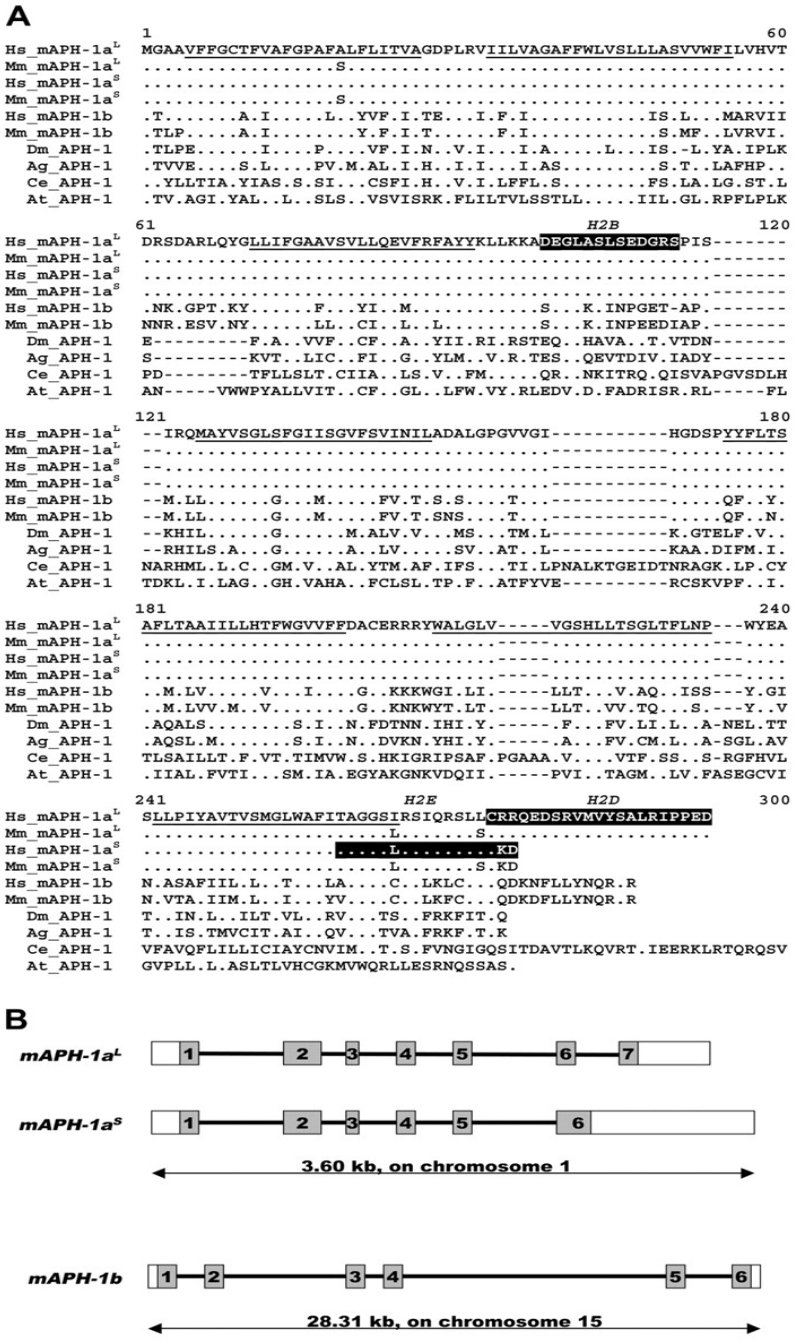


Figure 2.1. Protein sequences and gene structures of mAPH-1. *A*, predicted amino acid sequences of *Homo sapiens* (*Hs*) and *Mus musculus* (*Mm*) mAPH-1, and *Drosophila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*), *Caenorhabditis elegans* (*Ce*), and *Arabidopsis thaliana* (*At*) APH-1 orthologs. GenBank™ accession numbers are: AAH01230 (Hs_mAPH-1a^L), AAH08732 (Hs_mAPH-1a^S), AAH20905 (Hs_mAPH-1b), AAH24111 (Mm_mAPH-1a^L), AAH12406 (Mm_mAPH-1a^S), BAB22004 (Mm_mAPH-1b), AAF51212 (Dm_APH-1), EAA14158 (Ag_APH-1), CAA16282 (Ce_APH-1), and AAL36063 (At_APH-1). The Ce_APH-1 is predicted to contain an additional 17-amino acids at the carboxyl terminus, which were trimmed in this alignment. mAPH-1a^L and mAPH-1a^S are identical except at the carboxyl terminus. mAPH-1a is ~50% identical to mAPH-1b. *Dots* depict identical amino acid residues to Hs_mAPH-1a^L. Putative transmembrane domains are *underlined*. Amino acid sequences of the peptides used as antigens for generating the mAPH-1a antisera are *highlighted. B*, the gene structures and alternative splicing mechanism of human *mAPH-1*. Exons are *boxed. Shading* indicates coding sequences. *Empty boxes* indicate non-coding regions. The *lines* between exons indicate introns. *mAPH-1a* maps to a region of chromosome 1 that has been previously linked to an Alzheimer's disease susceptibility locus in a full genome scan (32). *mAPH-1b* maps to chromosome 15.

vivo (Fig 2.2 and 2.3). H2D-2 affinity purified antibody raised against the c-terminal 21 amino acids of mAPH-1a specifically recognizes mAPH1aL and co-immunoprecipitation experiments reveal endogenous mAPH1aL, presenilin-1NTF, and nicastrin co-precipitated with presenilin-1 CTF from rat brain membrane lysates. In reverse immunoprecipitations nicastrin and presenilin-1 CTF co-precipitated with endogenous mAPH-1aL from rat brain membrane lysates. As control preimmune serum failed to precipitate known γ-secretase complex members. These biochemical observations in addition to the reported *C.elegans* genetic linkages reveal that mAPH-1 specifically interacts with nicastrin and the presenilin heterodimers (Fig 2.2).

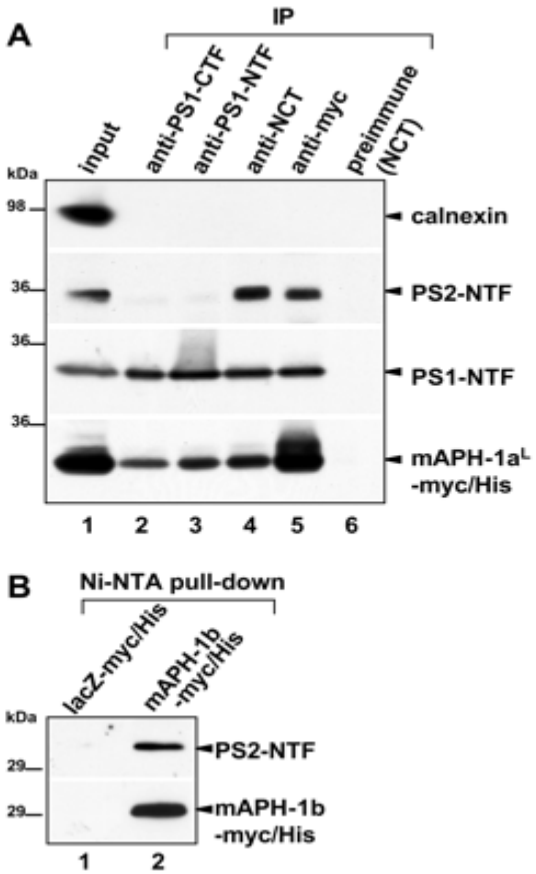


Figure 2.2. Recombinant mAPH-1 interacts with nicastrin and the presenilin heterodimers. *A*, HEK293 cells stably expressing mAPH-1a^{L}-Myc-His were solubilized in 1% CHAPSO and immunoprecipitated using the antibodies as indicated at the *top*. The immunoprecipitation products were resolved on SDS-polyacrylamide gel and the immunoblots were investigated with antibodies to calnexin, presenilin-2 NTF, presenilin-1 NTF, or Myc epitope. The *lane* labeled *input* represents ~20% of the starting CHAPSO lysate utilized for the immunoprecipitation. *B*, 1% detergent extract of HEK293 cells stably expressing LacZ-Myc-His (*lane 1*) or mAPH-1b-Myc-His (*lane 2*) were subjected to Ni-NTA-agarose pull-down experiments. The pull-down products were resolved on SDS-polyacrylamide gel, and the immunoblots were investigated with antibodies to presenilin-2 NTF (*top panel*) or Myc epitope (*bottom panel*). Please note that the comparable amount of LacZ-Myc-His can be detected by anti-Myc in *lane 1*. The LacZ-Myc-His band was cut off from the panel because of its larger size.

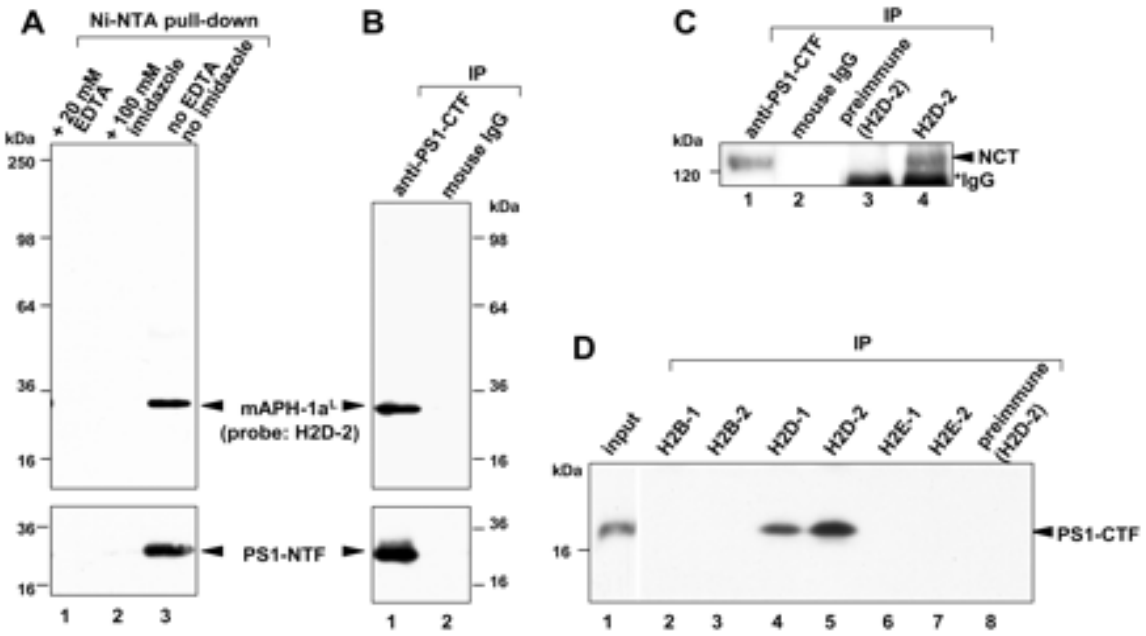


Figure 2.3. Endogenous mAPH-1 interacts with nicastrin and the presenilin heterodimers *in vivo*. *A*, 1% digitonin or 1% CHAPSO (data not shown) extracted proteins from HeLa or HEK293 (data not shown) cells expressing mAPH-1a^L-Myc-His were subjected to Ni-NTA-agarose pull-down experiments in the lysis/washing buffer plus 20 mM EDTA (*lane 1*), 100 mM imidazole (*lane 2*), or no EDTA/imidazole (*lane 3*). The pull-down products were resolved on SDS-polyacrylamide gel, and the immunoblots were investigated with antibodies to mAPH-1a^L (affinity-purified H2D-2, *top panel*) and presenilin-1 NTF (*bottom panel*). *B*, rat brain membranes were solubilized in 1% digitonin and immunoprecipitated using the mouse monoclonal anti-presenilin-1 CTF antibody (MAB5232, Chemicon) (*lane 1*) or normal mouse IgG (*lane 2*). The immunoprecipitation products were resolved on SDS-polyacrylamide gel, and the immunoblots were investigated with antibodies to mAPH-1a and presenilin-1 NTF as in *panel A*. *C* and *D*, rat brain membranes were solubilized in 1% digitonin and immunoprecipitated using the antibodies as indicated at the *top*. The immunoprecipitation products were resolved on SDS-polyacrylamide gel, and the immunoblots were investigated with antibodies to nicastrin (*C*) or presenilin-1 CTF (*D*). The *asterisk* indicates IgG. The *lane* labeled *input* represents ~20% of the starting digitonin lysate utilized for the immunoprecipitation.

RNA interference studies in HeLa cell lines were initiated to further study the role of endogenous mAPH-1 in γ-secretase activity (Fig 2.4). Twenty one nucleotide siRNAs were used to inactive endogenous APH-1. Reverse transcriptase PCR was used to detect down regulation of mAPH1. Specificity of inactivation can be compared between downregulation of the highly homologous mAPH-1a versus mAPH-1b. The inactivation of mAPH-1 in mammalian cells resulted in a dramatic reduction of presenilin

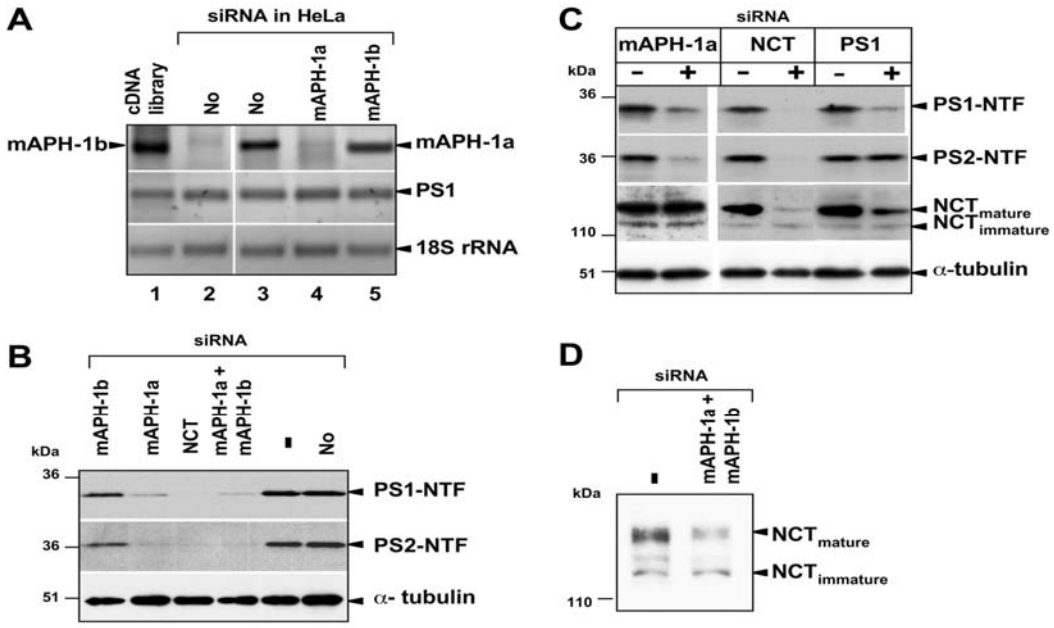


Figure 2.4. Inactivation of endogenous *mAPH-1* results in reduced levels of steady-state presenilins and nicastrin. *A*, cDNAs prepared from the indicated HeLa cells (no siRNA-treated cells, *lanes 2* and *3*; *mAPH-1a* or *mAPH-1b* siRNA-treated cells, *lanes 4* and *5*) were subjected to PCR using primer pairs for *mAPH-1b* (*top left panel, lanes 1* and *2*) or *mAPH-1a* (*top right panel, lanes 3-5*). Please note that *mAPH-1b* mRNA expression is low in HeLa cells (*top left panel, lane 2*). The human glioblastoma U118-MG cDNA library was used as a control sample for the *mAPH-1b* primers (*top left panel, lane 1*). All of the samples were also amplified using primer pairs specific for *presenilin-1* (*middle panel*) and 18 S rRNA (*bottom panel*) to control for equal PCR amplifications. *B*, HeLa cells were transfected with either the indicated siRNA duplexes, sense oligonucleotides (−), or no oligonucleotide (*No*) harvested 72 h post-transfection and immunoblotted for presenilin-1 NTF, presenilin-2 NTF, and α-tubulin with the appropriate antibodies. *C*, HeLa/$APP_{Swedish}$ stable cells were subjected to transfection with either the appropriate sense oligonucleotide (−) or siRNA duplexes (+) corresponding to *mAPH-1a*, *nicastrin*, or *presenilin-1*, respectively, and immunoblotted for presenilin-1 NTF, presenilin-2 NTF, nicastrin, and α-tubulin. *D*, Sense oligonucleotides (−) or *mAPH-1a* plus *mAPH-1b* siRNA-treated samples from *panel B* were probed with anti-nicastrin antibody. Please note that a lower percentage SDS-polyacrylamide gel was used for this panel as compared with the rest of the panels. Please also note that the presenilin levels are lower in the sample treated with *mAPH-1a* plus *mAPH-1b* siRNAs (*panel B*) than in the sample treated with *mAPH-1a* siRNAs alone (*panels B* and *C*).

heterodimers but had no effect on control proteins. This downregulation was assessed to be at the protein level since RNA levels of presenilin remained steady. Similarly, nicastrin inactivation results in the downregulation of steady state levels of presenilin heterodimers. Overall levels of nicastrin RNA do not change again pointing to an importance of mAPH-1 in the stability of the γ-secretase complex at the protein level. Inactivation of mAPH-1 does not show as dramatic of an effect on nicastrin. This may be

explained due to the inefficiencies associated with the knockdown approach, or by postulating that nicastrin : mAph-1 interactions may precede those of presenilin maturation. Thus a greater effect is observed on presenilin heterodimer formation. Together with the observed data from C. elegans genetics, co-immunoprecipitation as well as RNA interference, these data support mAPH-1 as a functional and physical protein component that is critical for γ-secretase complex physiologically (Fig 2.4).

We went on to test if mAPH1 like presenilin and nicastrin may modulate the γ-secretase cleavage of substrate APP. A marked increase in the immediate γ-secretase substrates C83 and C99, respectively the α- or β-cleaved carboxyl-terminal fragments of APP are observed upon inactivation of endogenous mAPH-1. Inactivation was carried out in HeLa cells that stably express full-length APP Swedish protein. Similar results as expected are observed upon inactivation of nicastrin or presenilin. No significant changes in total levels of full-length APP or control proteins are observed. Furthermore, immunoprecipitation with Aβ-specific antibodies from cell lines where mAPH-1 was down regulated revealed decreased levels in total Aβ secreted into the condition media when compared to control cells (Fig 2.5).

The mAPH-1a inactivated cells were also used to test the production of APP intracellular domain. A previously established and sensitive cell-based assay that indirectly measures a product of γ-secretase (Cao and Sudhof 2001). In the transactivation assay, a luciferase target gene is co-expressed with a chimeric protein where Gal4VP16 is fused at the c-terminal of a substrate. Gal4 is the yeast DNA-binding

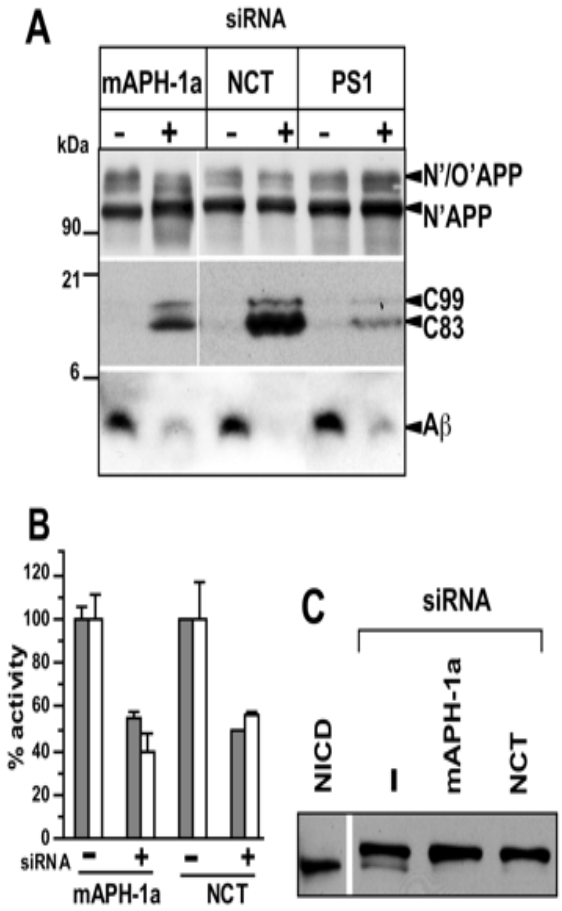


Figure 2.5. mAPH-1 modulates γ-cleavage of APP and Notch. *A*, the siRNA-treated samples from Fig. 4*C* were blotted for full-length APP, C99, and C83 using an APP-CTF polyclonal antibody, whereas the levels of Aβ in the 16-h conditioned media were analyzed as described under "Experimental Procedures." *B*, γ-secretase-mediated cleavage of C99-GV and NΔE-GV luciferase reporter assays. HeLa cells treated with either the appropriate sense oligonucleotides (−) or siRNA duplexes (+) corresponding to either *mAPH-1a* or *nicastrin* were subjected to transfection with β-galactosidase and either C99-GV, NΔE-GV, or a plasmid containing the Gal4 DNA-binding domain and assayed for luciferase reporter activity. Data collected were normalized to the β-galactosidase activity and then converted to fold activation over Gal4. The *graph* represents a typical experiment performed in quadruplets and is shown as percent activity (means ± S.E.) where 100% activities for the C99-GV (*gray bar*) and the NΔE-GV (*open bar*) assays are 150- and 100-fold activation over Gal4, respectively. *C*, HeLa cells treated with either sense oligonucleotides (−), *mAPH-1a* siRNA duplex, or *nicastrin* siRNA duplex were subjected to transfection with carboxyl-terminal Myc-tagged NΔE. Samples were lysed and blotted with anti-Myc 9E10. The migration position of the cleaved NICD is shown on the *left*.

domain and the viral VP16 acts as the transcriptional activating domain. C99-GV is able to activate the luciferase target gene only upon γ-secretase release of the intracellular domain from the membrane and to trafficked to the nucleus. Luciferase activity was reduced in mAPH-1a and nicastrin knockdown cells lines when compared to control cell lines. mAPH-1 like the known components is essential for APP cleavage. Correlating to these observations are those of Notch transactivation assay. siRNA-mediated inactivation of mAPH1 resulted in a reduction of a chimeric NΔE-GV substrate from activating luciferase. Hela cells treated with RNA interference specific for APH-1 and nicastrin resulted in reduced cleavage and nuclear trafficking of NΔE. These results are similar to

those observed upon down regulation of presenilin. Like presenilin and nicastrin, mAPH-1 is critical for the γ-secretase cleavage of substrates in mammalian cells (Fig 2.5).

mAPH-1 meets the criteria of a γ-secretase complex component that was previously described in the introduction. Endogenous mAPH-1 interacts with nicastrin and presenilin. mAPH-1 is required for the maturation of presenilin into an active enzyme. From observations of immediate substrate levels and our cell based assays mAPH-1 is critical for the cleavage of substrates such as Notch and APP. The steady state level observations of nicastrin and presenilin upon APH-1 inactivation support a model of assembly that may require an initial sub complex of APH-1 and nicastrin early in the secretory pathway. Overexpression studies of APH-1 alone or in conjunction with presenilin and nicastrin do not boost γ-secretase activity, thus proving insufficient to account for "limiting cellular factor" hypothesis. In this regards, the additional protein that was required was identified as PEN-2, using similar procedures as described for mAPH-1 (Luo, Wang et al. 2003).

Future studies that address aspects of how these four components assemble, mature, and modulate γ-secretase activity will remain of interest. Individual roles of each in carrying out this very unusual enzymatic activity should prove helpful in further discerning its role throughout development and disease.

Discussion

Efforts in screening additional genes essential for notch/glp-1 signaling in *C. elegans* proved fruitful. At the genetic level two additional components of the complex, termed APH-1 and PEN-2 were identified (Francis, McGrath et al. 2002). Our data presented here demonstrates that these proteins act as cofactors at the biochemical and cellular level for the γ-secretase. We have reported that the mammalian APH-1 (mAPH-1) proteins physically associate with nicastrin and the presenilin heterodimers in vivo, are required for maturation of nicastrin and presenilin to the active enzyme complex, and are indispensable for the intramembrane proteolysis of both APP and Notch.

The unusual intramembrane aspartyl protease activity of γ-secretase was elucidated before the true nature of the enzyme complex. The data reported in this chapter yields insights into the minimum constituents of the gamma-secretase complex. These new key components are critical in the maturation of previously identified presenilin and nicastrin. For presenilin, endoproteolysis is required for entry into core active complex. For nicastrin, complex N-linked glycosylation associated with trafficking through the secretary pathway and may facilitate its incorporation into the active enzyme. Transiently or exogenously expressed nicastrin or presenilin protein entry into the active enzyme complex requires stable or significant overexpression. These observations have led to difficulties in properly examining the functional effects of engineered mutations in these proteins using the normal cell transfection methods. The new components, mAPH-1 and PEN-2 facilitate the conversion of presenilin and nicastrin from immature to mature

forms, and these conversions correlate with the formation of the active γ-secretase complex. Only upon exogenously expressing the four components together do we overcome the limitations associated with formation and stabilization of the active γ-secretase (Luo, Wang et al. 2003).

Having, contributed to the understanding of the composition of the γ-secretase complex, little was known about the assembly process and function of the individual components. This is partly due to the difficulties described in analyzing each component individually in the absence as well as presence of its other partners. Future studies by us and others as described in the next chapters would shed light on the cooperative regulation of these partners in the assembly, trafficking and formation of the active γ-secretase. With the interdependence of all four components, understanding the individual functions may raise new strategies to inhibit γ-secretase and further our knowledge behind the molecular mechanisms employed by this highly unusual enzyme.

Chapter 3

A GXXXG motif in Aph1 is critical for assembly of the γ-secretase complex

Attributions: In this chapter, I made significant intellectual contributions as well as was involved in the initial cloning and characterization of APH-1. Some experiments were performed collaboratively and others where my involvement was purely indirect. It is difficult and may be inaccurate to attribute each aspect of the work individually except to acknowledge all authors in the publications below and particularly Sheu-Fen Lee and Hongqia Li whom I worked very closely with during the earlier years of my graduate studentship. I thank Drs Philip Thomas and Christian Wigley for their willingness to discuss aspects of this project early in my graduate school tenure.

Publications: Data in this chapter is published in Lee *et al.,* Journal of Biological Chemistry 2004.

Abstract

The amyloid precursor protein (APP) and the Notch cell surface receptor undergo a remarkably unusual cleavage within its transmembrane. The nature of the enzyme that is responsible for this aspartyl protease activity called γ-secretase remained unknown. In the previous chapter, genetic screens coupled to bioinformatics performed on the growing genome sequence databases, demonstrated that γ-secretase is a protein complex composed of presenilin, nicastrin, Aph-1 and PEN-2. The complete set of protein components required to generate Aβ as well as transduce Notch intracellular domain dependent signaling from their respective precursors had been identified. Presenilins fall within a novel class of polytopic aspartyl proteases and constitutes the active site of this enzyme. We and others hypothesized that other components may be required for the assembly, maturation and stabilization of the complex. Specifically, a conserved transmembrane Gly123 in *C.elegans* displayed a notch loss of function phenotype. In this chapter we show that such mutations in mammalian APH-1a disrupt the physical interaction with nicastrin, presenilin and PEN-2. Similarly, the mammalian homolog mutant also reduced γ-secretase activity in intramembrane proteolysis. We found that the fourth conserved transmembrane of Aph-1 contains a helix-helix interaction GXXXG motif and is essential for the stable association of Aph-1a with presenilin, nicastrin, and Pen-2. Thus, Aph-1 plays a critical role in the initial assembly and subsequent maturation and maintenance of the active γ-secretase complex.

Introduction

γ-Secretase remained a unique and elusive aspartyl protease. Its active site resides within the hydrophobic environment of the lipid bilayer. First identified for generating Aβ, this fragment accumulates to form the central hallmark of plaques in Alzheimer's disease (AD). Generation of Aβ takes place when γ-secretase cleaves its ectodomain shed precursor (APP), a type I glycoprotein that may serve as a cell surface receptor. In a similar fashion, Notch a cell surface receptor, cleaved by this same enzyme critical for various cell fact decisions during embryogenesis through adulthood. Notch transduces its signal through the release of NICD. Over the years, the number of single transmembrane substrates that may be cleaved intramembranously has grown. Potentially, suggesting that the normal function of γ-secretase may be to serve as a signaling hub. Thus, efforts in identifying and characterizing γ-secretase complex components results in broader progress towards understanding functions of normal biology and disease.

In the last chapter I described efforts by Dr. Gang Yu's lab as well as many other groups in identifying components that are critical for mediating this enzymatic activity. Substantial evidence supports that the presenilin proteins constitute the active site of the protease. Genetic inactivation of PS or its orthologues in *C.elegans, D. melanogaster* and *M. musculus* result in the disruption of γ-secretase-mediated processing of Noth and/or APP. Two conserved intramembrane aspartates have proven to be essential in the hydrolysis of

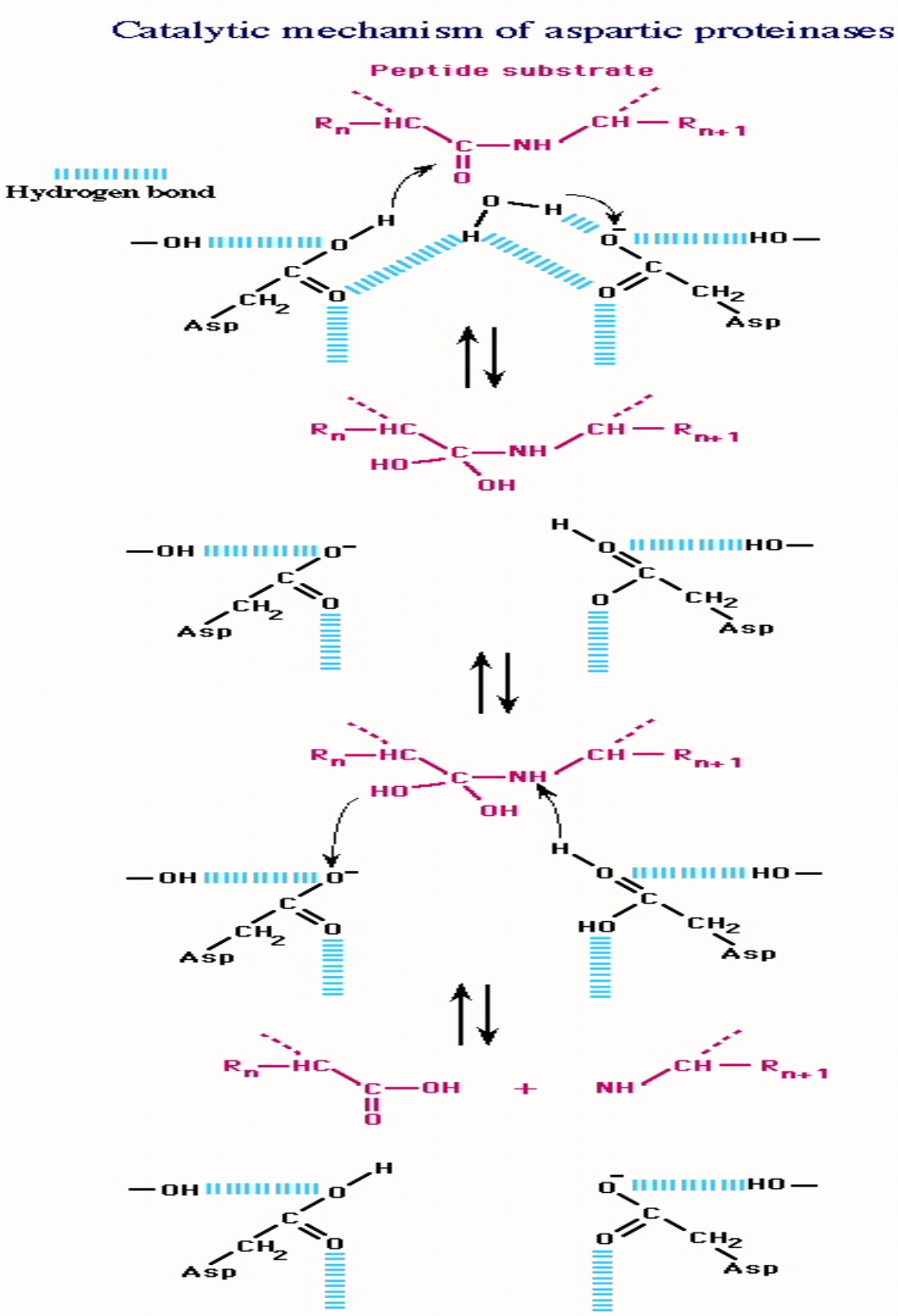


Figure 3.1. Aspartyl Proteases Mechanism. Initial mechanisms to describe how aspartyl proteases cleave this bond were aimed at demonstrating a covalent acyl intermediate. However, it became clear that no covalent intermediate exist in this class of proteases, unlike the closely related family of the serine proteases. Instead, the different pKis of the two catalitic aspartates lead to one acting as a general acid catalyst to protonate the carbonyl oxygen, and the other acting as a general base to pull the proton from water. This permits direct nucleophilic attack by the water oxygen to the carbonyl carbon to form an amide dihydrate intermediate. This then breaks down by the conjugate forms of the two aspartates to form the cleaved products.

an amide bond (Wolfe, Xia et al. 1999). These two aspartates are conserved in all members of the presenilin gene family, and transition-state analogue inhibitors bind directly to presenilin. Such data implicates presenilin as the central component providing

the necessary active site but not necessarily as the only one (Fig 3.1). In fact, genetic inactivation of nicastrin, Aph1, and PEN-2 in *C.elgans* or *D. melanogaster*, results in a Notch phenotype that is indistinguishable from knockout of PS (Francis, McGrath et al. 2002; Goutte, Tsunozaki et al. 2002). Little was known about the functional role these additional components play in the normal assembly, maturation and maintenance of the γ-secretase complex.

In the last chapter we showed that in addition to the genetic screens that had identified Aph-1 as a member of the γ-secretase complex, the mammalian form is functionally conserved and physically associates with presenilin and nicastrin. The function of Aph-1 within this complex remained unclear. Initial evidence supported a role for PEN-2 in the endoproteolysis of presenilin (Gu, Chen et al. 2003; Takasugi, Tomita et al. 2003). Whereas APH-1 and nicastrin may act as a scaffold forming an initial subcomplex, before it can completely mature to a state of an active enzyme (Lee, Shah et al. 2002). Interestingly, the initial genetic screens for Aph-1 reported the mutation Gly(123) to an aspartic acid within the fourth transmembrane region of *C. elegans* (**mutant or28**) as inhibiting notch signal transduction. Bioinformatics of this region revealed to us that this glycine residue in C. elegans Aph-1 was conserved during evolution and corresponds to G122 in mammals. Moreover, we found two additional Gly residues (Gly126 & Gly130) downstream of the mutant glycine residue within this transmembrane region forms a putative helix-helix interaction GXXXG motif (where X represents any amino acid) (MacKenzie, Prestegard et al. 1997). Such a motif is generally accepted as a major determinant in transmembrane helix-helix protein interactons leading

us to suspect that these residues may be critical for γ-secretase complex assembly (Popot and Engelman 2000; Fleming and Engelman 2001).

Our efforts in this chapter were aimed at revealing the specific role for Aph-1 in the scaffolding and assembly of the γ-secretase complex. With substantial evidence and characterization of residues required for catalytic activity, we had now identified a conserved membrane helix-helix interaction GXXXG motif in Aph-1 as a critical determinant for the specific components of the complex early in its maturation. Approaches such as this one, provide a framework for which the molecular mechanisms involved in assembly, maturation and maintenance of this remarkably unusual intramembrane protease may be further understood. This portion of our study contributes a functional role for a recently identified component of the complex. Understanding the individual functions of each component may raise new therapeutic approaches to inhibit the γ-secretase and further extend our understanding behind the molecular mechanisms employed by this highly unusual enzyme.

Methods

cDNA Constructs, Cell Lines —Human full-length *APH-1a*^{L} and *PEN-2* cDNAs were obtained as described. Subsequent site-directed mutagenesis studies were performed using the QuikChange kit (Stratagene), and the identities of all clones were confirmed by DNA sequence analyses. Cells were maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum with 5% CO_2 at 37 °C. For transient expression, plasmids were transfected into the appropriate cell lines in 10-cm dishes using LipofectAMINE 2000, and samples were collected 48 h after transfection. HEK293 cells stably or transiently transfected with Myc/His-tagged APH-1a^{L} and its glycine mutants were used to evaluate the interaction of APH-1 with presenilin, nicastrin, and PEN-2.

Antibodies and Chemicals—The antibodies used in this study include anti-Myc monoclonal antibody 9E10 (American Type Culture Collection); anti-Myc polyclonal antibody A14 (Santa Cruz Biotechnology); anti-FLAG antibody M2 (Sigma); antibodies against presenilin-1-(21–80) (Chemicon International, Inc.), presenilin-1-(263–378) (Chemicon International, Inc.), and presenilin-2-(7–24) (Oncogene); antibody against nicastrin-(693–709) (Sigma); anti-PEN-2 antibody PNT2; and antibody against the hemagglutinin (HA) epitope (Santa Cruz Biotechnology). Inhibitors of γ-secretase activity (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester and *N*-(2-naphthoyl)-Val-phenylalaninal (inhibitor IV)) were from Calbiochem.

Coprecipitation Studies—Co-immunoprecipitations were performed as described previously. Briefly, proteins extracted using lysis buffer (1% digitonin or 1% CHAPSO in 50 mM sodium phosphate (pH 8.0), protease inhibitors (Roche Applied Sciences) and 300

mM NaCl) were pre-absorbed with preimmune serum and combined with anti-Myc antibody A14 overnight and then with protein A-agarose beads for 2 h at 4 °C. The beads were washed four times for 15 min each with lysis buffer. Immunoprecipitated proteins were eluted with 0.1 M glycine HCl (pH 2.5) and 0.25% detergent, neutralized with 1.0 M Tris, and subjected to Western blot analysis. Ni-NTA-agarose affinity pulldown experiments were performed as described previously in 1% digitonin or 1% CHAPSO lysis buffer. Each binding experiment contained equal amounts of proteins with similar expression of Myc/His-tagged proteins.

γ-*Secretase Activity Assay*—The appropriate cell lines were assayed for γ-cleavage of Notch using the NΔE-GV luciferase reporter as described in an adapted *in vitro* γ-secretase activity assay. The *in vitro* activity assay involved lysis of membrane proteins with 1% CHAPSO, 50 mM PIPES (pH 7), 5 mM $MgCl_2$, 5 mM $CaCl_2$, and protease inhibitors (Roche Applied Science) and immunoprecipitation of Myc-tagged proteins with immobilized anti-Myc antibody 9E10. The beads were then washed with CHAPSO lysis buffer and subjected to incubation at 37 °C in the presence of 0.25% CHAPSO, 50 mM PIPES (pH 7), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.0125% phosphatidylethanolamine, 0.1% phosphatidylcholine, and N100 substrate. This N100 substrate, which was overexpressed and purified from Sf9 cells, harbors Val^{1711}–Glu^{1809} of the mouse Notch-1 receptor and a carboxyl-terminal FLAG/His tag. The γ-secretase-cleaved N100-FLAG/His fragment was detected with anti-FLAG antibody.

Results

The GXXXG sequence of residues within membrane proteins is the most frequent of motifs associated with helix-helix interactions. This helix-helix interaction motif is structurally similar to the glycophorin A dimers, where two transmembrane helices associate to form a dimer stabilized by the GXXXG motif. The *C. elegans* mutation (**mutant or28**) Gly123 in the conserved transmembrane of APH-1 results in a notch/glp-1 loss of function phenotype. In an effort to further elucidate the function of this previously identified component of the γ-secretase complex, we analyzed here the ability of analogous human APH-1aL (wild type or G122D mutant) to interact with the presenilin endoproteolytic species. Our approach was to generate HEK293 cells stably expressing APH-1aL WT-Myc/His or APH-1aLG122D-Myc/His and examine cell lines with comparable levels of respective protein expression. Equivalent levels of endogenous presenilin fragments are observed in these stable cell lines. As previously reported, Ni-NTA agarose pull-down experiments, reveal APH-1aL WT-Myc/His was able to coprecipitate the endogenous presenilin-1 NTF. However, decreased levels of presenilin-1 NTF coprecipitated with APH-1aL G122D-Myc/His. Similarly, lower levels of presenilin-2 coprecipitated with APH-1aL G122D-Myc/His compared with significant amount of presenilin-1 and -2 fragments coprecipitated with APH-1aL-WT-Myc/His. These observations reveal that the G122D mutation disrupts the interaction between APH-1 and mature presenilin species (Fig 3.2). To test if human APH-1aL Gly122 is required for interaction with immature full length presenilin, we transiently overexpressed presenilin-1 holoprotein in the stable cell lines expressing APH-1aL WT-

Myc/His and APH-1aLG122D-Myc/His and performed Ni-NTA pull-down. Less full-length presenilin-1, as well as the previously observed endoproteolytic fragments, coprecipitated with APH-1aL-G122D-Myc/His compared with APH-1aLWT-Myc/His. The G122D mutations disrupt the ability of APH-1aL to interact with the mature presenilin endoproteolytic fragments and the immature presenilin holoprotein (Fig 3.3).

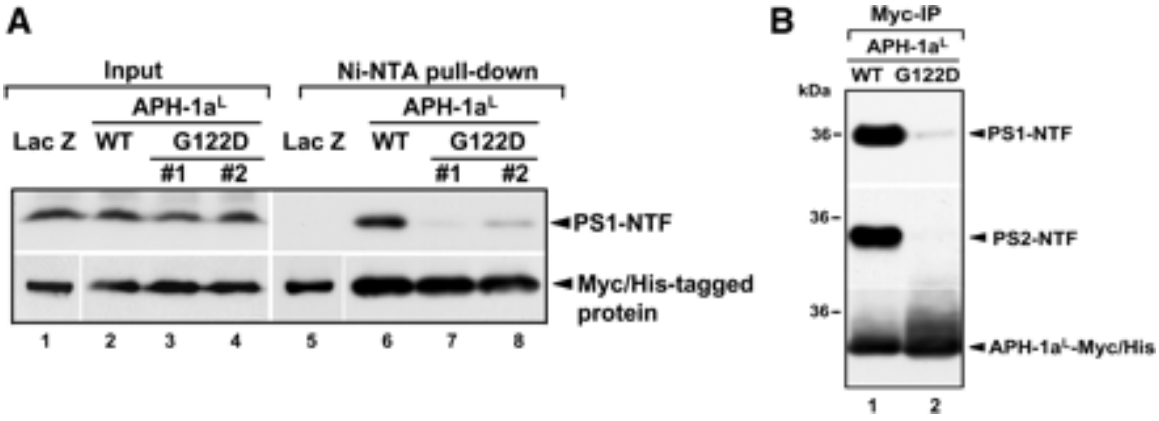


Figure 3.2. Mutation G122D in APH-1a^L disrupts the interaction of APH-1 with endogenous presenilin endoproteolytic fragments. *A*, equal amounts of protein extracts (*lanes 1–4*) of 1% digitonin-solubilized HEK293 cells stably overexpressing LacZ-Myc/His (*lanes 1* and *5*), APH-1a^{L}WT-Myc/His (*lanes 2* and *6*), or APH-1a^{L}G122D-Myc/His clone 1 (*lanes 3* and *7*) or clone 2 (*lanes 4* and *8*) were subjected to Ni-NTA pull-down experiments. The Ni-NTA pull-down products (*lanes 5–8*) were resolved by SDS-PAGE and probed with antibodies against presenilin-1 NTF (*PS1-NTF*) and the Myc epitope as indicated. Similar results were also obtained using 1% CHAPSO extracts. Note that the Myc/His-tagged proteins and presenilin-1 NTF were expressed at similar levels in each set of cells (*Input*) and that the sizes of the Myc/His-tagged proteins are different, but were aligned to save space. *B*, HEK293 cells stably overexpressing APH-1a^{L}WT-Myc/His (*lane 1*) or APH-1a^{L}G122D-Myc/His (*lane 2*) were solubilized in 1% CHAPSO and immunoprecipitated (*IP*) using anti-Myc antibody A14. The immunoprecipitated products were resolved by SDS-PAGE, and the immunoblots were investigated with antibodies to presenilin-1 NTF, presenilin-2 NTF (*PS2-NTF*), and the Myc epitope as indicated.

Analogously we tested the ability of immature and mature nicastrin to coprecipitate with APH-1a. Immature nicastrin is believed to be restricted to the early secretory pathway and is associated with full-length presenilin. While fully glycosylated mature nicastrin is associated with presenilin NTF/CTF fragments (Yang, Tandon et al. 2002) . APH-1aL-WT-Myc/His associates preferably with the immature forms of

nicastrin in cells overexpressing APH-1aL and the presenilin-1 holoprotein. Much less immature and mature nicastrin was co-retained with APH-1aLG122D-Myc/His compared with APH-1aL-WT-Myc/His. The WT stable cell lines also contained slightly more immature nicastrin than the mutant stably expressing cells (Fig 3.4). Thus the G122D mutation may also affect the ability of wild-type APH-1 to stabilize immature nicastrin (Hu and Fortini 2003). The mutation mediates the interaction of APH-1aL with both species of nicastrin during the initial assembly process of the γ-secretase complex.

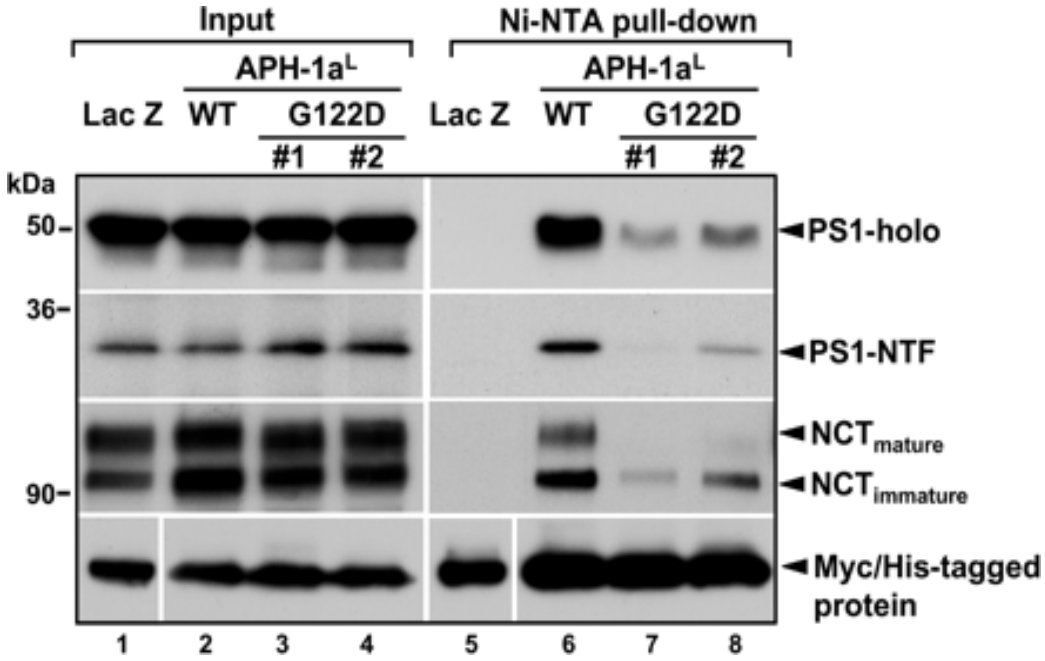


Figure 3.3. Mutation G122D disrupts the interaction of APH-1a^L with the presenilin holoprotein and nicastrin. Full-length presenilin-1 was transiently transfected into HEK293 cells stably overexpressing LacZ-Myc/His (*lanes 1* and *5*), APH-1a^{L}WT-Myc/His (*lanes 2* and *6*), or APH-1a^{L}G122D-Myc/His clone 1 (*lanes 3* and *7*) or clone 2 (*lanes 4* and *8*), and the cells were solubilized in 1% digitonin. After having determined that the presenilin-1 holoprotein and Myc/His-tagged proteins were expressed at similar levels in each set of cells, we subjected equal amounts of proteins (*lanes 1–4*) to Ni-NTA pull-down experiments. The resultant Ni-NTA pull-down products (*lanes 5–8*) were resolved by SDS-PAGE and probed with antibody against amino-terminal presenilin-1 to identify the presenilin-1 holoprotein (*PS1-holo*) and presenilin-1 NTF (*PS1-NTF*) and with antibodies against nicastrin (*NCT*) and the Myc epitope as indicated.

Similarly, the ability of G122D mutation to retain PEN-2 on Ni-NTA was tested. HA-tagged PEN-2 was transiently expressed in either of the stable cell lines. Overexpressed HAPPEN-2 retention was not disrupted by the G122D mutation, however endogenous PEN-2 was. Endogenous PEN-2 does not strongly coprecipitate with mutant APH-1L. The reasoning behind this may lie in the fact that the γ-secretase complex is very tightly regulated and failure of interaction between PEN-2 and APH-1 may be due to the disruption of earlier associations of APH-1 with presenilin and nicastrin (Fig 3.4).

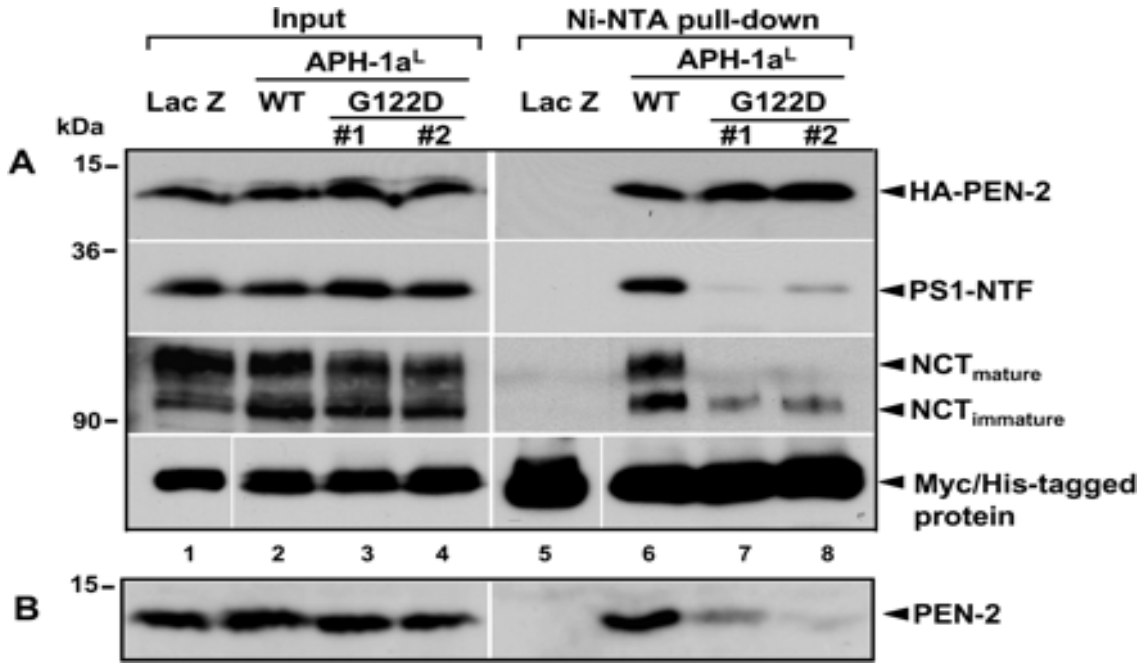


Figure 3.4. Mutation G122D disrupts the association of APH-1a^{L} with endogenous PEN-2, but not with overexpressed HA-PEN-2. *A*, cDNA encoding HA-tagged PEN-2 was transiently transfected into HEK293 cells stably expressing LacZ-Myc/His (*lanes 1* and *5*), APH-1a^{L}WT-Myc/His (*lanes 2* and *6*), or APH-1a^{L}G122D-Myc/His clone 1 (*lanes 3* and *7*) or clone 2 (*lanes 4* and *8*), and the cells were solubilized in 1% digitonin. Equal amounts of proteins (*lanes 1–4*) were subjected to Ni-NTA pull-down experiments. The resultant Ni-NTA pull-down products (*lanes 5–8*) were resolved by SDS-PAGE and probed with antibodies to the HA epitope, presenilin-1 NTF (*PS1-NTF*), nicastrin (*NCT*), and the Myc epitope as indicated. *B*, protein extracts of 1% digitonin-solubilized HEK293 cells stably overexpressing LacZ-Myc/His, APH-1a^{L}WT-Myc/His, or APH-1a^{L}G122D-Myc/His clone 1 or 2 were subjected to Ni-NTA pull-down experiments. The Ni-NTA pull-down products (*lanes 5–8*) were resolved by SDS-PAGE and probed with antibody against endogenous PEN-2. Note that endogenous PEN-2 (*lanes 1–4*) and the Myc/His-tagged proteins (data not shown) were expressed at similar levels in each set of cells and that equal amounts of proteins were used for Ni-NTA pull-down experiments.

Having established the G122D mutation in disrupting complex formation, we set out to observe the effects on γ-secretase activity by this mutation. The approach was to immunoprecipitate the γ-secretase complex containing either APH-1aL-WT-Myc/His or APH-1aLG122D-Myc/His from their respective CHAPSO-solubilized membranes and measure the ability of the immunoprecipitates to cleave a purified Notch substrate N100 in vitro. A stronger N100 cleavage in the immunoprecipitates containing wild-type APH-1aL was observed compared to the mutant. N100 proteolytic intracellular fragment was also sensitive to two different γ-secretase inhibitors. In summary, the G122D mutation in APH-1aL has an inhibitory effect on γ-secretase activity (Fig 3.5).

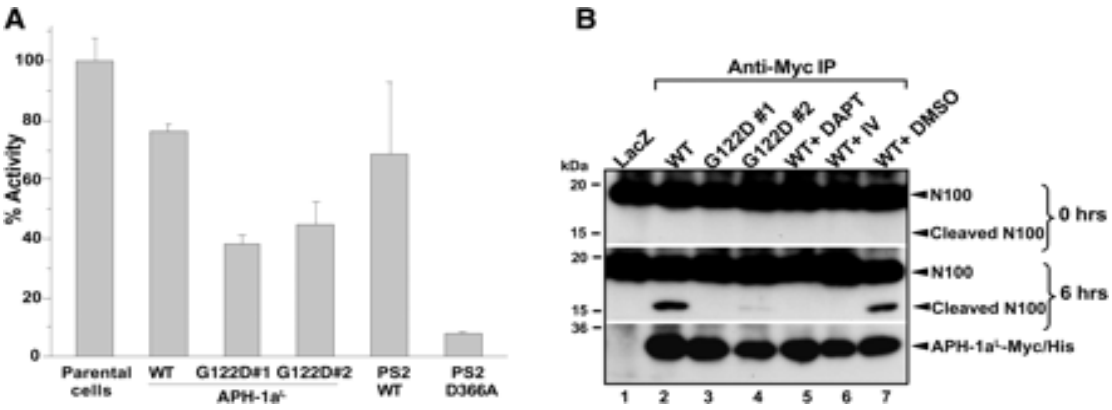


Figure 3.5. Mutation G122D in APH-1a^L inhibits γ-secretase activity. *A*, γ-secretase-mediated cleavage in NΔE-GV luciferase reporter assays. HEK293 cells (parental) or HEK293 cells stably expressing APH-1a^{L}WT-Myc/His or APH-1a^{L}G122D-Myc/His clone 1 or 2 were transfected with β-galactosidase together with either NΔE-GV or a plasmid containing only the Gal4 DNA-binding domain. Samples were assayed for luciferase reporter activity, and the data collected were normalized to the β-galactosidase activity prior to presenting the results as -fold activation over the Gal4-transfected samples. The graph, which represents an average of multiple experiments performed in duplicate, is shown as percent activity (means ± S.E.), where 100% activity for the NΔE-GV assay is 100-fold activation over the control Gal4 samples. HEK293 cells stably expressing wild-type (*PS2 WT*) and aspartate mutant (*PS2 D366A*) presenilin-2 were included as controls for the assay. *B, in vitro* γ-secretase-mediated cleavage of purified N100-FLAG/His. LacZ-Myc/His (*lane 1*), APH-1a^{L}WT-Myc/His (*lane 2*), or APH-1a^{L}G122D-Myc/His clone 1 (*lane 3*) or clone 2 *lane 4*) was extracted with 1% CHAPSO and subjected to immunoprecipitation (*IP*) with immobilized anti-Myc antibody 9E10. Washed immunoprecipitates were subjected to incubation with purified N100-FLAG/His at 37 °C for 0 and 6 h. Immunoprecipitate from APH-1a^{L}WT-Myc/His cells was also subjected to incubation with N100-FLAG/His in the presence of the γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (*DAPT*; *lane 5*), γ-secretase inhibitor IV (*IV*; *lane 6*), or Me_2SO (*DMSO*; *lane 7*). Samples were electrophoresed on SDS-polyacrylamide gel and probed with anti-FLAG antibody (*upper* and *middle panels*) or with anti-Myc antibody (*lower panel*).

The results so far suggest that the G122D mutation disrupts the ability of APH-1a to participate in the initial assembly process of the γ-secretase complex. It may be critical in the early assembly process of the γ-secretase complex and associate with and stabilize the active γ-secretase complex to directly modulate the intramembrane proteolysis of Notch. Nevertheless, a disruption in active complex formation and γ-secretase activity could be attributed to improper trafficking of APH-1aL within cells. To address this issue both immunofluorescence and linear glycerol density centrifugation were performed on stable cell lines. Immunoflouorescence revealed no observable trafficking defect with intracellular punctuate/vesicular staining patterns for both the wild-type and G122D mutant APH1-aL. In addition, analysis of the native state wild-type and G122D mutant APH-1aL through linear glycerol density centrifugation did not show any alterations. Both wild-type and mutant APH1aL were found to reside in high molecular mass glycerol density fractions. Although G122D may be incapable of tight association with

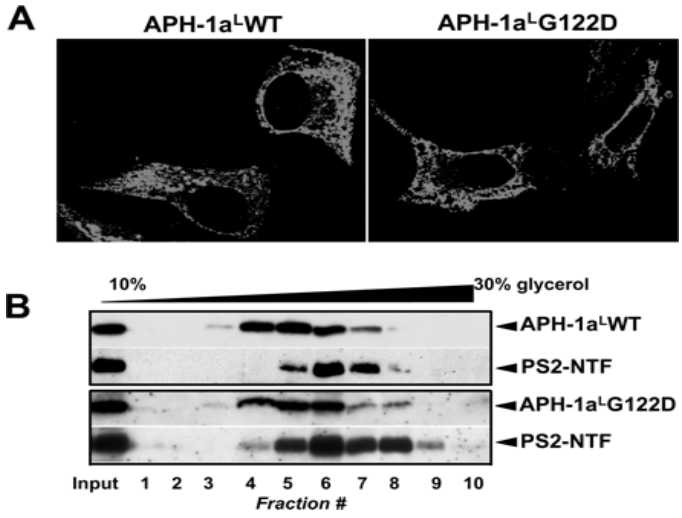


Figure 3.6. Mutation G122D does not result in major changes in the localization and native state of APH-1a^{L}. *A*, HEK293 cells overexpressing APH-1a^{L}WT-Myc/His or APH-1a^{L}G122D-Myc/His were probed with anti-Myc antibody A14 and processed for immunofluorescence analyses. *B*, cell lines stably expressing APH-1a^{L}WT-Myc/His or APH-1a^{L}G122D-Myc/His were fractionated over a 10–30% linear glycerol velocity gradient. A 1-ml fraction from each set of cells was collected from the top of the gradient and analyzed with either anti-Myc antibody 9E10 or anti-presenilin-2 NTF antibody (*PS2-NTF*).

presenilin and nicastrin, the glycerol density centrifugation reveals some degree of association however, inconsistent with forming an active complex (Fig 3.6).

APH-1aL G122D mutant falls within a conserved GXXXG motif important in transmembrane helix-helix associations (Fig 3.7). These conserved transmembrane glycines may form a sequence arrangement motif that resembles these transmembrane protein interactions. The conserved sequence arrangement known as the GXXXG motif was originally identified in a single TMR of glycophorin A and required for its dimerization (Fleming and Engelman 2001). It is recognized as a high affinity transmembrane helix-helix binding motif for many other membrane proteins including aquaporin-1, ErbB-4, ATP synthase, and the anion-selective membrane channel VacA.

Figure 3.7. Conserved G*XXX*G motif in the APH-1 family of proteins. The primary structure alignment of the fourth putative transmembrane helix of APH-1 family members and the TMR of human glycophorin A (*GpA*) is shown. Residue numbers correspond to human APH-1a. The glycine residues in the G*XXX*G motif believed to be critical for helix-helix interaction are shown in *boldface*, and those examined in this study are shown in *black boxes*. Note that the APH-1 G*XXX*G motif could be extended to potentially include (A/S)*XXX*G*XXX*G*XXX*(G/A). The G*XXX*G motif in glycophorin A is shown in a *gray box*, and the important residues in the motif are *underlined*. Species and GenBank™/EBI accession numbers are as follows: *Homo sapiens* (*Hs*), AAH01230and AAH20905 *Mus musculus* (*Mm*), AAH24111and AAH12406 *Danio rerio* (*Dr*), AAM88325 *Anopheles gambiae* (*Ag*), EAA14158 *Drosophila melanogaster* (*Dm*), AAF51212 *C. elegans* (*Ce*), CAA16282 and *A. thaliana* (*At*), AAL36063

To test if the ^{122}GXXXG^{126} sequence is a bona fide transmembrane interaction motif of APH-1, we set out to determine if disrupting the G122D APH-1aL in the γ-secretase complex is not dependent exclusively on changing the glycine to charged side chains such as aspartic acid. APH-1 glycine residues were replaced with either the nonpolar residues alanine or proline and tested for there ability to interact with endogenous presenilin, nicastrin, and PEN-2. Ni-NTA agarose pull-down experiments, transiently transfected with APH-1aLG122A-Myc/His coprecipitated with similar amounts of the other three γ-secretase complex components. Therefore, the effects of Gly122 mutations are not solely dependent on the aspartates. GXXXG motif glycine substitutions with alanine have proven to be conservative in APH-1aL as well as other proteins that contain such a motif. Less steric clashes may occur upon replacing the glycine with alanine compared with other residues and thus may be less disruptive for the γ-secretase complex.

To establish that APH-1aL ^{122}GXXXG^{126} is part of an authentic transmembrane interaction motif, we replace the conserved glycine of APH-1aL at position 126 with either alanine or leucine. Compared with WT APH-1aL, G126A mutation reduces the interaction of APH-1aL with endogenous presenilin-1 NTF, nicastrin and PEN-2. The G126L mutation in APH-1aL abolishes the interaction to the same extent as the G122P or Gd122D mutation. Mutations of similar conserved glycine resides in APH-1aL outside the GXXXG motif did not significantly affect the interaction of APH-1aL with other γ-secretase components (Fig 3.8).

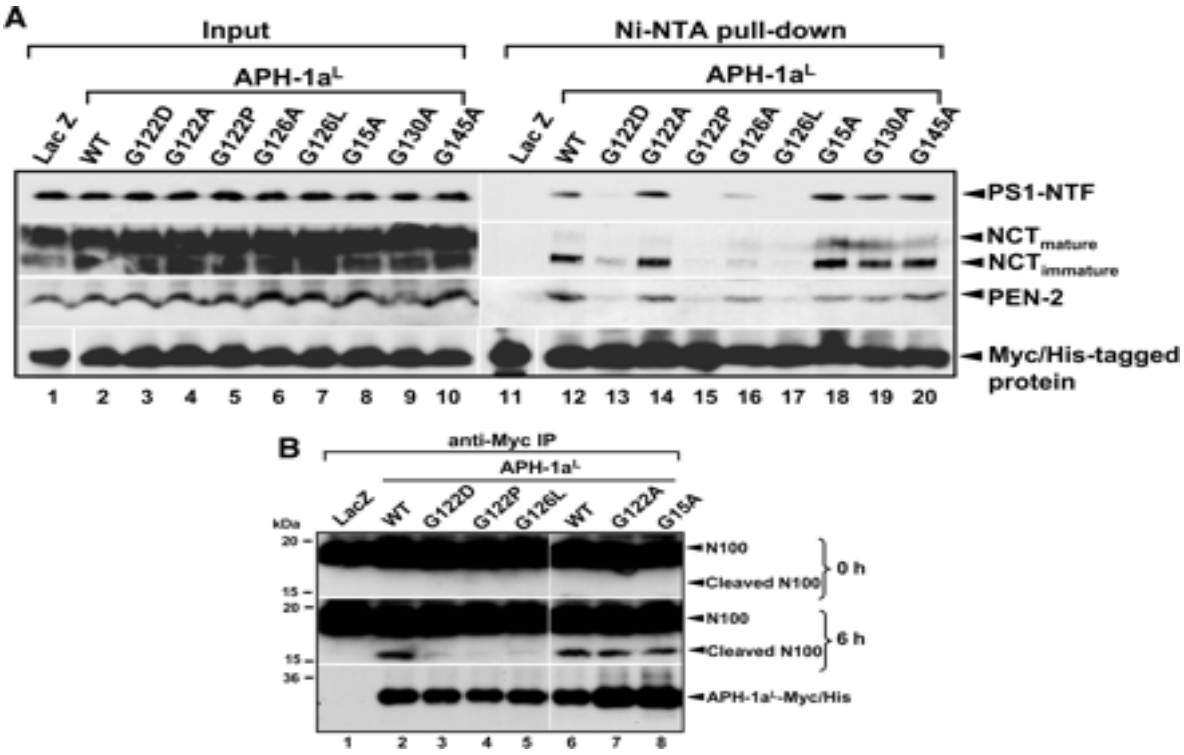


Figure 3.8. Mutations of the critical glycine residues in the ^{122}G*XXX*G^{126} sequence disrupt the assembly and activity of the γ-secretase complex. *A*, HEK293 cells transiently transfected with LacZ-Myc/His (*lanes 1* and *11*), APH-1a^{L}WT-Myc/His (*lanes 2* and *12*), or APH-1a^{L}-Myc/His harboring a single mutation of the glycine residue indicated (*lanes 3–10* and *13–20*) were lysed in 1% digitonin. After determining that similar amounts of the Myc/His-tagged proteins, endogenous presenilin-1, nicastrin, and PEN-2 were expressed in each set of cells, we subjected equal amounts of proteins (*lanes 1–10*) to Ni-NTA pull-down experiments, and the resultant products (*lanes 11–20*) were resolved by SDS-PAGE and probed with antibodies to presenilin-1 NTF (*PS1-NTF*), nicastrin (*NCT*), PEN-2, and the Myc epitope as indicated. *B*, cell lines stably expressing LacZ-Myc/His (*lane 1*), APH-1a^{L}WT-Myc/His (*lanes 2* and *6*), or APH-1a^{L}-Myc/His harboring a single mutation of the glycine residue indicated (*lanes 3–5* and *7–8*) were processed as described in the legend to Fig. 4*B* and subjected to incubation with N100-FLAG/His at 37 °C for 0 and 6 h. Samples were electrophoresed on SDS-polyacrylamide gel and probed with anti-FLAG antibody (*upper* and *middle panels*) or with anti-Myc antibody (*lower panel*). *IP*, immunoprecipitation.

Next we observed the glycine mutations role in γ-secretase activity. We generated stable HEK293 cell lines for G15A, G122A, G122P, G126L, in addition to cells stably expressing the WT and G122D Myc/His. Upon immunoprecipitation of the γ-secretase complex containing either wild-type or mutant APH1aL-Myc/His from their respective CHAPSO-solubilized membranes, they were measured for in vitro activity. G122D, G122P and G126L reduced N100 cleavage, whereas G122A and G15A did not have a noticeable effect on N100 cleavage. As the G122A reconstitutes and assembles into the γ-secretase complex, it also maintains proteolytic activity. In summary, these observations are consistent with the conserved Gly122 and Gly126 in APH-1aL as critical residues that make up a highly specific GXXXG motif required for the assembly and activity of the multimeric γ-secretase complex.

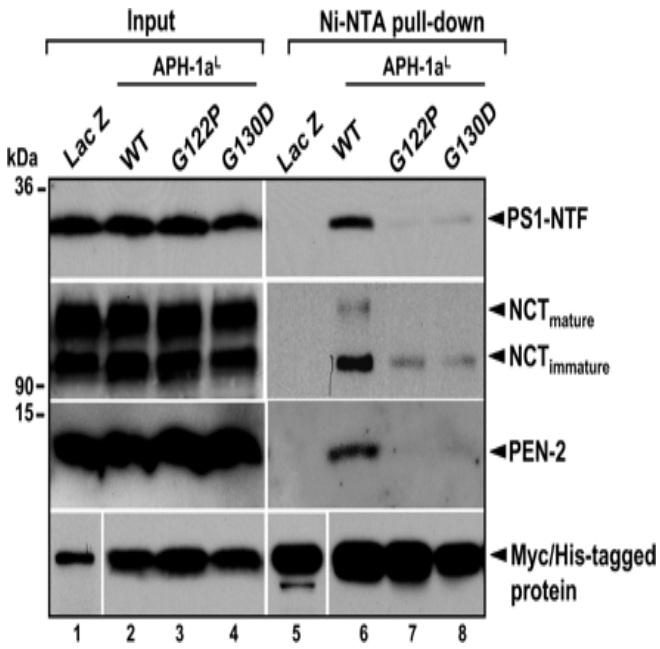


Figure 3.9. Mutation G130D disrupts the interaction of APH-1a^{L} with the other γ-secretase components. Equal amounts of protein extracts (*lanes 1–4*) of 1% digitonin-solubilized HEK293 cells transiently transfected with LacZ-Myc/His (*lane 1*), APH-1a^{L}WT-Myc/His (*lane 2*), APH-1a^{L}G122P-Myc/His (*lane 3*), or APH-1a^{L}G130D-Myc/His (*lane 4*) were subjected to Ni-NTA pull-down experiments, and the resultant products (*lanes 5–8*) were resolved by SDS-PAGE and probed with antibodies to presenilin-1 NTF (*PS1-NTF*), nicastrin (*NCT*), PEN-2, and the Myc epitope as indicated.

Sequence comparison and α-helical wheel analysis suggested that residues surrounding APH-1a^{L} Gly^{122} and Gly^{126}, such as Ala^{118}, Ser^{129}, Gly^{130}, and Ser^{133}, may

also be important for transmembrane helix-helix association. In particular, Gly^{130} is highly conserved, with its equivalent residue in *C. elegans* or *Arabidopsis thaliana* APH-1 being alanine. Previous experiments showed that the G130A mutation did not affect the ability of APH-$1a^L$ to associate with the other γ-secretase subunits suggests that the conserved alanine-for-glycine substitution at position 130 may be tolerated at position 122. To test whether Gly^{130} (like Gly^{122} and Gly^{126}) is also a critical residue for transmembrane interaction, we replaced Gly^{130} with aspartate and tested the ability of G130D mutant APH-$1a^L$ to interact with the other γ-secretase components. Pull-down experiments, revealed much less endogenous presenilin-1 NTF, mature and immature nicastrin, and PEN-2 coprecipitated with transiently expressed APH-$1a^L$G130D-Myc/His compared with APH-$1a^L$WT-Myc/His. These findings suggest that Gly^{130} (like Gly^{122} and Gly^{126}) is critical for mediating interactions in the multimeric γ-secretase complex, providing evidence that the glycophorin A-like helix-helix binding motif in putative TMR-4 of APH-$1a^L$ is longer (^{122}G*XXX*G*XXX*G^{130}). Future studies may elucidate the exact sequence and structural requirements for this critical motif in the assembly and activity of the γ-secretase complex (Fig 3.9).

Discussion

The observations reported in this chapter are the first ones to define a helix-helix interaction among the four components of the γ-secretase complex. In terms of molecular weight, it is estimated that at least half of the complex is imbedded as (at least 18 TMR) within the hydrophobic lipid bilayer. Molecular interactions amongst these TMRs may be

essential for the assembly, maturation, and ultimately cleavage of substrates. Upon identification of all the components required to form an active γ-secretase complex, intense interest was drawn towards how this unusual enzyme is assembled. In this chapter, we report the identification of conserved Gly122, Gly126 and Gly130 in transmembrane 4 of APH-1. These Gly residues are separated in a fashion that we recognized is part of the GXXXG motif. First identified in glycophorin A, the GXXXG motif has been widely accepted as a critical determinant in transmembrane helix-helix interactions. The Gly residues are critical in that (1) they provide a surface on the TMR for packing (2) permit TM helix proximity and (3) promote entropy effects (Fig 3.10). Glycine residues allow alpha helix to make intimate contact which is further stabilized by van der Walls packing.

In this study we show that mutations of Gly122, Gly126, and Gly130 in the GXXXG motif of APH-1 disrupt γ-secretase complex formation. *C. elegans* **or28** mutant harbors the equivalent to Gly122D site directed mutant in mammals (Goutte, Tsunozaki et al. 2002). The mutant displays the recessive loss-of-function notch/glp-1 phenotype which can now be explained as due to a disruption in the early assembly of the γ-secretase complex. In this study, we proposed the GXXXG motif of APH-1 as a major and highly specific docking and packing site for the assembly of the γ-secretase complex early in the secretory pathway.

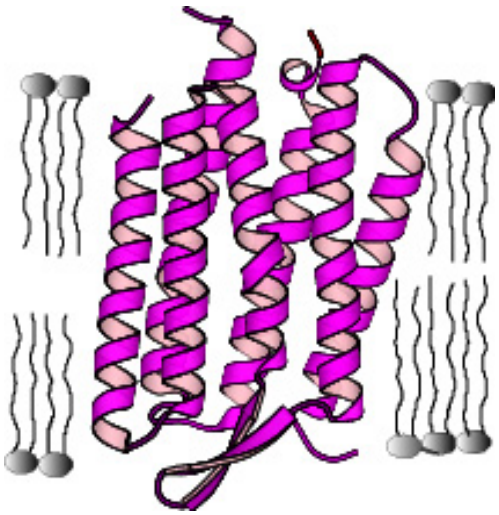


Figure 3.10. The GXXXG motif. Experimentally found as a motif that is responsible for stabilizing helix-helix interactions in both membrane and water soluble proteins. The two glycine reisdues are found at i and i + 4 positions, and cover one helical turn. Periodicity analysis of such sequence in membrane proteins has found this motif as 32% above expectation in alpha helices. The oligomerization is enabled by the glycine's side chain that is the smallest of all amino acids, and allows for a tight packing of helices. GXXXG motif promotes backbone-to-backbone contacts thereby stabilizing helix-helix interactions in soluble as well as membrane proteins.

The GXXXG motif muations in APH-1 disrupt its interaction with the mature and immature forms of presenilin and nicastrin. Thus, affecting γ-secretase activity. The GXXXG motif binding is hypothesized to play an important role in the docking and packing of APH-1 in the early phases of complex assembly. How exactly APH-1 GXXXG may regulate the assembly and maturation of γ-secretase complex remains to be discerned. In several reported cases, GXXXG motifs facilitate homooligomerization resulting in the speculation that APH-1 exists as a dimer within the γ-secretase complex. On the other hand, interactions with other components or the complex can not be ruled out. In fact the presenilin family proteins harbor several conserved glycine residues that may potentially constitute a transmembrane GXXXG motif. Recent reports suggest that APH-1 and nicastrin interact to form a stable subcomplex intermediate. Presenilin may not be able to co-isolate with the GXXXG mutant of APH1 because of defects in the direct binding of TMR of APH1 and nicastrin.

Our findings here reveal a specific role for APH1 in the scaffolding of the γ-secretase complex. The GXXXG motif in APH-1 may constitute a critical determinant for the specific interaction among the components that are needed for γ-secretase activity. Studies support a mechanism where by the biosynthetic process of the gamma-secretase complex, begins with nicastrin and Aph-1 form a heterodimeric intermediate complex and bind to the C-terminal region of PS, serving as a stabilizing scaffold for the complex. Pen-2 is then recruited into this trimeric complex and triggers endoproteolysis of PS, conferring gamma-secretase activity. Recent data support that the two-thirds of TMD4 of PS1, including the conserved Trp-Asn-Phe sequence, are required for its interaction with Pen-2 (Watanabe, Tomita et al. 2005) (Kim and Sisodia 2005). As the γ-secretase complex mature Pen-2 may contribute to the activation of the gamma-secretase complex by directly binding to presenilin (Fig 3.11).

Future studies into the role of motifs such as the GXXXG and NF will shed light on the TM protein interaction required within the lipid bilayer for the assembly, maturation and maintenance of the γ-secretase complex. With four distinct protein components and at the minimum 18 transmbrane helixes many aspects of the component interactions as well as associated changes required to perform cleavage within the membrane still remains uncharted territory. Identification of additional motifs may add to the therapeutic strategies that target Alzheimer's diseases and related disorders. Recently, a nucleotide-binding site within the transmbrane regions of the γ-secretase complex was proposed to be involved in differentially influencing substrate selectivity (Fraering, Ye et al. 2005). For example, ATP was shown to directly increase the generation of APP

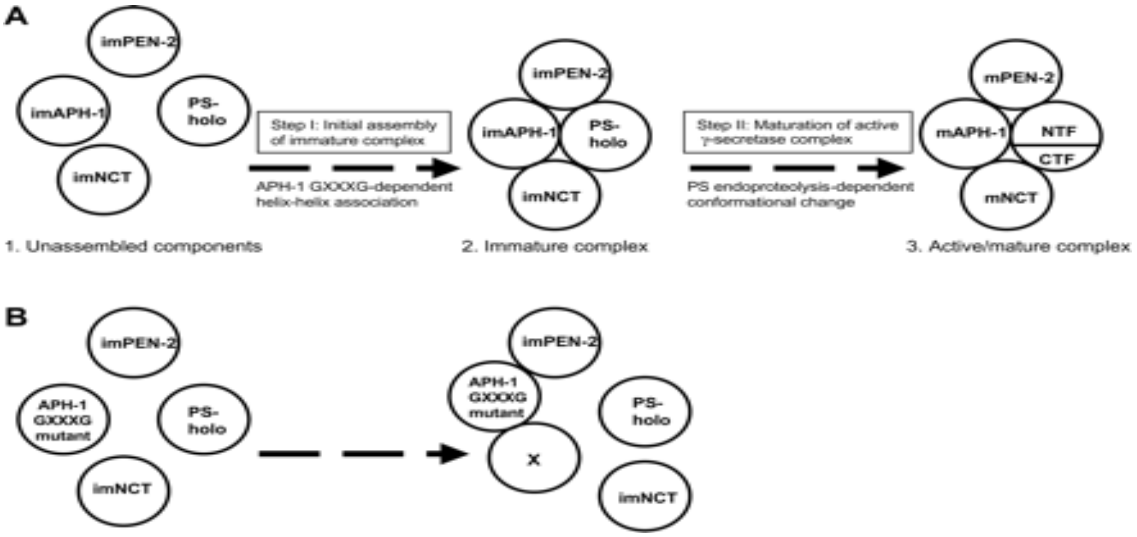


Figure 3.11. Model of the G*XXX*G-dependent helix-helix association in modulating the assembly and activity of the γ-secretase complex. *A*, in this simplified model, APH-1, PEN-2, presenilin, and nicastrin (*NCT*) exist in at least three major states during the initial assembly and subsequent maturation of the active γ-secretase complex: 1) unassembled immature components, 2) immature complex (probably labile in most cells), and 3) active/mature complex. Our data and results from other groups (10–16) suggest that APH-1 functions as a molecular scaffold for the γ-secretase complex. G*XXX*G-dependent helix-helix association plays an essential role in the initial assembly of the immature complex (*Step I*) and is also required for stabilizing the active mature complex (*Step II*). Transition of the immature complex to the mature complex is probably associated with a presenilin (*PS*) endoproteolysis-triggered conformational change (*Step II*). Note that multiple substeps likely exist within the two major steps described here. *B*, mutations of the G*XXX*G motif prevent APH-1 from performing its scaffolding role in the initial assembly of the immature complex (and consequently in maturation of the active complex). G*XXX*G mutant APH-1 may recruit additional molecule(s) (marked *X*), self-oligomerize, or adopt an alternative folding. In agreement with this view, we found that mutant APH-1 could still associate with overexpressed (presumably unassembled/immature) PEN-2 and that mutant APH-1 existed in a high molecular weight complex without associating with presenilin and nicastrin. It should be noted that the cognate site of the APH-1 G*XXX*G motif is unknown and that APH-1, PEN-2, presenilin, and nicastrin could have multiple contacts with one another. It should also be noted that the stoichiometry of the γ-secretase complex is unclear and that γ-secretase subunits could potentially form homodimers or homo-oligomers. For simplicity, only one copy of each subunit was depicted. *Im*, immature; *m*, mature; *PS-holo*, presenilin holoprotein.

intracellular domain and Aβ, but not the generation of Notch intracellular domain by a purified protease complex. Interestingly, domains in soluble proteins containing GXXXG motifs in conjugation with Rossmann folds strongly influence binding with nucleotides (Kleiger and Eisenberg 2002). Conserved GXXXG motifs have been found in the transmembrane regions of APP and one may hypothesize these as target sites that may further influence aspects of γ-secretase complex assembly as well as establish aspects of substrate selectivity.

Chapter 4

Nicastrin serves as a γ-secretase substrate receptor

Attributions: In this chapter, I made a significant intellectual and hands on contribution. I took a leading role on a majority of questions and problems associated with understanding the major role of Nicastrin in the γ-secretase complex. Some experiments were performed collaboratively. I received advice and guidance from Dr. Thomas Südhof and Dr. Gang Yu in sub group meetings regarding this project. Drs. Charles Dan III and Haydn Ball collaborated and provided generous advice/resources on aspects of nicastrin ectodomain substrate binding site interactions as well as incorporating native chemical ligation strategies to modify free amine of substrates. Dr. Katsuhiko Tabuchi provided the nicastrin knockout cell lines used in some aspects of this study. Lab members Dr. Sheu-Fen Lee established the baculovirus system and Dr. Yi-Heng Hao collaborated with me on several aspects of the antibody and chemical blocking of the free amine of substrates. Dr. Cong Yu was also involved in confirming and establishing certain binding assays described in the proposal. Quincey LaPlant rotated through the lab under my guidance and participated in reconstituting and analyzing γ-secretase complexes in vitro. Beyond this, it is difficult and may be inaccurate to attribute each aspect of the work individually except to acknowledge all past and present members of Dr. Gang Yu's lab as well as students who rotated through our lab that I worked very closely with.

Publications: Data in this chapter is published in Shah *et al.,* Cell 2005.

Abstract

γ-Secretase catalyzes the intramembrane cleavage of amyloid precursor protein (APP) and Notch after their extracellular domains are shed by site-specific proteolysis. Nicastrin is an essential glycoprotein component of the γ-secretase complex but has no known function. In addition, γ-secretase appears to have a very broad substrate specificity. This chapter addresses two interrelated questions that are central to the molecular mechanisms of γ-secretase-mediated RIP. First, it attempts to address the biochemical function of nicastrin in the γ-secretase complex. Second, it attempts to show how the recognition of a broad range of substrates may be mediated. Specifically, we show that the ectodomain of nicastrin binds the new amino terminus that is generated upon proteolysis of the extracellular APP and Notch domains, thereby recruiting the APP and Notch substrates into the γ-secretase complex. Chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduce the binding and cleavage of substrate by γ-secretase. The results indicate that nicastrin is a receptor for the amino-terminal stubs that are generated by ectodomain shedding of type I transmembrane proteins. Our data are consistent with a model where nicastrin presents these substrates to γ-secretase and thereby facilitates their cleavage via intramembrane proteolysis.

Introduction

Ectodomain shedding, a process where ectodomains are proteolytically released has been now attributed to a growing list of cell surface proteins. These proteins are structurally and functionally diverse and include membrane anchored growth factors, cytokine receptors as well as ectoenzymes, and membrane adhesion receptors. A prerequisite, ectodomain shedding event, of many group I transmembrane receptors results in a subsequent second proteolytic event within the hydrophobic lipid membrane environment. The regulated intramembrane proteolysis (RIP) of these substrates is believed to release the membrane tethered, biologically active, cytoplasmic domains that may enter the nucleus as transcriptional regulators (Brown, Ye et al. 2000).

RIP is a well conserved, widely employed, signaling mechanism that is fundamentally controlled by proteases. Regulated intramembrane proteolysis (RIP) of the various type-I membrane proteins, including the amyloid-beta precursor protein (APP) and the Notch receptor, are catalyzed by an aspartyl protease called**,** γ-secretase. The γ-secretase is an unusual multimeric protein complex, consisting of three polytopic transmembrane proteins; presenilin (containing the critical aspartates for catalytic activity), recently identified APH-1 and PEN-2, as well as a single spanning glycoprotein nicastrin that associate to form an active high molecular weight core complex.

Recent developments have revealed a large number of putative type-I membrane proteins that may participate in cell signaling through γ-secretase dependent RIP events.

γ-Secretase appears to have very broad substrate specificity. The only known prerequisites for γ-secretase substrates are (1) type I membrane proteins and (2) shedding the bulk of the extracellular domains from the full-length precursor proteins. Thus, one critical unanswered question concerning γ-secretase mediated RIP is how the γ-secretase complex recognizes its many heterogeneous substrates which seem to have no specific amino acid sequences or substrate recognition domains common amongst them. One factor important in initial substrate recognition may be the orientation as well as origination of short extracellular domain after the primary processing event.

Current evidence supports presenilin and its endoproteolytic fragments as the catatlytic subunit of the γ-secretase complex (Wolfe, Xia et al. 1999). However, the precise function for Aph-1, Pen-2, and nicastrin in the complex and in intramembrane proteolysis remains unclear. Evidence for Aph-1 and Pen-2, points to a role in the assembly and maturation process of the complex. Unlike the polytopic membrane proteins presenilin, Aph-1 and Pen-2, nicastrin is a type-1 membrane glycoprotein with a large and unique ectodomain. In addition to evidence for nicastrin ectodomain playing a role in structural assembly, maturation, and activity of γ-secretase, preliminary data also suggested a putative role for the nicastrin ectodomain functioning as a receptor for γ-substrates.

1) Nicastrin can bind to the immediate substrates of (C100 & N99) gamma secretase under conditions that disrupt the core complex (Chen, Yu et al. 2001).

2) A portion of nicastrin ectodomain has sequence similarity to mono-zinc aminopeptidases, however no catalytic activity has been detected to date. Alterations, in these conserved regions of nicastrin ectodomain have effects on intramembrane cleavage of γ-substrates, particularly APP (Yu, Nishimura et al. 2000).

3) Studies using inhibitor affinity chromatography of the complex co-purifies the γ-substrates and points to a yet to be identified pre-docking site distinct form the active site of the complex (Esler, Kimberly et al. 2002).

We hypothesized that nicastrin, the only component of the core complex which contains a large extracellular domain, is a protein that may serve as a receptor for substrates. In this chapter we now show, that the APP or Notch derived γ-secretase substrates (C100 and N99) stoichiometrically, directly, and functionally interact with nicastrin. This interaction is mediated by the extracellular N-terminal stub of the substrate and the ectodomain of nicastrin. We find that a conserved glutamate (Glu333) within the extracellular aminopeptidase domain of nicastrin to be essential for substrate docking and γ-secretase catalytic activity. Chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduce the binding and cleavage of substrate by γ-secretase. These results define an interaction site between the extacellular domains of both the substrate and nicastrin and suggest a putative function for nicastrin as a receptor for γ-secretase substrates.

Methods

cDNA constructs, antibodies, & cell lines – Human cDNAs were used unless indicated otherwise. Wild-type and mutant cDNAs for γ-secretase subunits and substrates were cloned into baculoviral, bacterial, and mammalian expression vectors. Wild-type and mutant Nct-his, PS1, APH-1aL-His, HA-PEN-2, FLAG-PEN-2, C99-Flag, N100-FLAG/His and His-Syt1 cDNAs were cloned into pFASTBac and baculoviruses were generated using the Bac-toBac Baculovirus Expression System (Invitrogen). pFASTBac/C99-Flag encodes C-terminally Flag-tagged C99 with a Met followed immediately by the β-secretase cleaved APP C-terminal fragment. pFASTBac/nFlag-C99 encodes a C99-derived polypeptide in which the seven residues of C99 after Met were replaced by the Flag epitope (DYKDDDDDK). pFASTBac/N100-FLAG/His encodes Val1711-Glu1809 of mouse Notch-1 and a C-terminal Flag/His tag. The bacterial expression construct pET21b/C99-Flag was a gift from Drs. M.J. LaVoie, D.J.Selkoe, and Y.M. Li (Esler, Kimberly et al. 2002). To generate the construct for Cys-C99 (pHV-Cys), the His-tag and tobacco etch virus (TEV) recognition site sequence from pHis-parallel was amplified and mutated via PCR to generate an NdeI/BamHI product with an internal NsiI site, changing the final Gly of the TEV recognition site to Cys. The fragment was then subcloned suing the same sites into pET21b followed by insertion of C99-Flag as an NsiI/XhoI fragment. pCMV-IgG fusion constructs for mammalian cells were generated to contain the following sequences of various proteins fused to the Fc fragment of human IgG: nicastrin residues 1-50, nicastrin residues 1-669, full length α2δ1, and APP residues 1-205. pSGHV0-Nct(ECD) encodes nicastrin residues 34-669 in frame with the human

growth hormone (hGH), an octahitidine tag (H8), and a cleavage site for TEV protease (Leahy, Dann et al. 2000; Dann, Hsieh et al. 2001). Wild-type and mutant nicastrin cDNAs in pcDNA6-V5/His and a cDNA for the APP signal peptide-fused C99 (SpC99) in pcDNA3 were generated as previously described in (Lee, Shah et al. 2002). *Nicastrin*-deficient embryonic fibroblasts were obtained from *nicastrin* knockout mice, which will be reported elsewhere (K.T., G.Y., and T.S., unpublished data). Antibodies used in this portion of the study include ati-sera for APH-1aL, PEN-2, PS1, and Nct (Yu, Nishimura et al. 2000; Lee, Shah et al. 2002; Lee, Shah et al. 2004), anti-cleaved Notch-1 (Val1744) antibody (Cell Signalling), anti-APP cytoplasmic domain (anti-APP-CTD), anti-biotin, anti-human IgG, anti-Flag (Sigma), anti-His (Qiagen), anti-HA (Santa Cruz), and anti-fluorescein/Oregon Green (Molecular Probes) antibodies. Standard methods such as site-directed mutagenesis, transfection, Western blotting, and affinity precipitation were performed as described (Yu, Chen et al. 1998; Yu, Nishimura et al. 2000; Lee, Shah et al. 2002; Lee, Shah et al. 2004). All experiments in this paper were performed at least four times with multiple replications. The GraphPad Prism software was used for statistical analysis and graphing.

Protein Purification Methods - Purification of recombinant γ-secretase and its substrates (N100-Flag/His, C99-Flag, and nFlag-C99) from insect cell membranes and the nicastrin ectodomain from conditioned media of mammalian cells. Membranes from Sf9 cells infected with baculoviruses were solubilized in 1% Trition X-100 or 0.5% CHAPSO using a procedure similar to that for mammalian cells. The active recombinant γ-secretase and its subunits were purified from the membrane extracts by Ni-NTA affinity chromatography, glycerol gradient fractionation, anti-Flag affinity

chromatography, and HiTrap Q ion-exchange chromatography on an FPLC system (Amersham). γ-Secretase substrates N100-Flag/His, C99-Flag, and nFlag-C99 were purified using Ni-NTA or anti-Flag affinity chromatography. IgG-fusion proteins were purified on protein A resins from conditioned media of HEK293 cells stably expressing the IgG-fusion cDNAs. Untagged nicastrin ectodomain was expressed in Chinese hamster ovary (CHO) cells as a fusion protein with the human growth hormone, an octahistidine tag, and a TEV protease recognition site. hGH-Nct(ECD) was initially purified by Ni-NTA immobilized metal affinity chromatography from conditional media of the CHO cells. The fusion protein was then cleaved by TEV protease, and untagged nicastrin ectodomain was further purified by MonoQ anion exchange chromatography and gel filtration to near homogeneity (Leahy, Dann et al. 2000). N-formylated C99-Flag (fC99) was purified on anti-Flag beads from detergent extracts of Δ*acrAB E. coli* strain AG100A(DE3) (a gift from Dr. R.T. Sauer) transformed with pET21b/C99-Flag and induced by 1 mM IPTG plus 2 μg/ml actinonin for 5 hr at room temperature. Cys-C99 was expressed in *E. coli* as a fusion protein with an N-terminal His_6 tag and a recognition site for tobacco etch virus (TEV) protease as well as a C-terminal Flag tag. The fusion protein purified on Ni-NTA beads was cleaved with His-tagged TEV protease. Purified Cys-C99 was obtained after Ni-NTA depletion of the undigested fusion protein and TEV protease. Chemical synthesis of fluorescein- and biotin-LC-Gly-Gly-thioester and their ligation to the α-amino group of Cys-C99 was performed using native chemical ligation procedures.

In vitro γ-secretase assay - N100-Flag/His, nFlag-C99, C99-Flag, Cys-C99, and their N-terminally modified variants were incubated at 37°C with recombinant γ-secretase

purified from Sf9 cells or with cell-free γ-secretase from HeLa cells in a reaction buffer containing 0.25% CHAPSO, 50 mM PIPES (pH 7.0), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.0125% phosphatidylethanolamine, and 0.1% phosphatidylcholine. A typical 50 µl reaction uses 25 µg γ-secretase preparation and 0.1 µg substrate. AICD-Flag or NICD′-Flag/His was analyzed on Tris-Tricine SDS-polyacrylamide gels using anti-APP-CTD, anti-Flag, or anti-cleaved Notch-1 (Val1744) antibody. γ-Secretase cleavage of APP or C99 was also analyzed in HEK293 or fibroblast cells using the luciferase reporter gene assay system as described. The fluorogenic reporter peptide mimicking the γ-secretase-cleavage sites of APP was assayed as reported (Farmery, Tjernberg et al. 2003).

Biochemical Analyses – Edman protein sequencing and mass-spectrometry (MS) services were provided by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center. The first five amino acids from five cycles of N-terminal protein sequencing are: C99-Flag from E. coli, MDAEF; Cys-C99 from E. coli, CMDAE; N100-Flag/His from SF9 cells, VKSEP; NICD'-Flag/His generated in vitro, VLLSR; Nct-His form Sf9 cells, NSVER; Nct(EDC)-IgG from HEK293 media, NSVER. Immunoprecipitation and protein A and Ni-NTA affinity pull-down experiments were performed as previously described except that 1% Trition X-100 and higher salt concentration were used for investigating interaction of γ-secretase substrates and nicastrin. For the Flag-peptide pre-absorption or elution experiments, saturating amounts of Flag peptide were used. For in vitro γ-secretase assays, addition of lipids (e.g phospatidylcholine, phospatidylethanolamine, or phophatidylinositol) is absolutely necessary for purified recombinant γ-secretase from Sf9 cells but is not required for cell-free γ-secretase from crude HeLa or fibroblast cell membranes. γ-

Secretase activity can be reconstituted in proteoliposomes when CHAPS is removed from a mixture of cholesterol and phosphatidylcholine by dialysis and ultracentrifugation.

Native Chemical Ligation Material – 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydoxybenotriazole (HOBt) and tert-butoxycarbonyl (t-Boc) – protected amino acids were obtained from Peptides International (Lousiville, KY). N,N-diisopropylethylamine (DIEA), diisopropylcarodiimide (DIC), 5,6-carboxyfluorescien and triisoproplysilane were purchased from Aldrich. Methylbenzhydrylamine (MBHA) resin and d-biotin-amidocarproic acid (biotin-LC) was obtained from Anaspec (San Jose, CA). N-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), HPLC grade acetonitrile and dichloromethane (DCM) were obtained from Fisher. Hydrogen fluoride (HF) was purchased from Matheson Gas (Cucamonga, CA).

Synthesis of Fluorescein-Gly-Gly-Thioester and Biotin-LC-Gly-Gly-Thioester Derivatives – t-Boc Leu (1mmol) was activated with 0.9 mmol of HBTU/HOBt in the presence of 2 mmol of DIEA and coupled for 1 hr to 0.3 mmol of MBHA resin. The Boc group was removed following a 30 min treatment with 50% TFA in DCM. The TFA salt was neutralized by washing with 5% DIEA in DCM for 5 mins. Next, 1 mmol of S-trityl marcaptopropionic acid was activated with 0.9 mmol HBTU/HOBt and 2 mmole of DIEA. The coupling was allowed to continue for 3hrs. The resulting trityl-associated mercaptopropionic acid-leucine resin was ready for manual solid phase peptide synthesis (SPPS) after the S-trityl group is removed with a 1 x 1 min and 1 x 10min treatment with 50% TFA in DCM and 1% triisopropylsilane. After SPPS the

N-terminal t-Boc protecting group was removed with a 30 min using 50% TFA in DCM. A three-fold excess 5,6 carboxyfluorescien or biotin-LC was dissolved in NMP and activates with DIC and HOBt and added to the Gly-Gly-thioester resin. Coupling was monitored using the quantitative ninhydrin test. The derivative or unmodified thioester peptide was cleaved from the resin support with anhydrous hydrogen fluoride to yield a peptide that could be used for native ligation. Analytical reversed-phase HPLC was performed on a Waters 717 system using either a C4 or C18 Vydac column (5μm, 150 x 4.6 mm). Semi-preparative HPLC was conducted on a Waters 600 system using a C18 Vydac column (10μm, 250 x 10mm). Buffer A was water/0.045% TFA and buffer B was acetonitrile/0.036% TFA. A linear gradient was applied from 0% B to 100% B in either 30 mins at a flow rate of 1ml/min (analytical) or 120 mins at a flow rate of 3 ml/min (semi-preparative). Detection was at 220 nm. Matix-assisted laser-desorption ionization (MALDI) mass spectrometry (MS) was performed on a Micromass L/R reflectorn instrument (Waters Corp., Milford MA) by using α-cyano-4-hydroxycinnamic acid (Agilent) as a matrix.

Native chemical ligation reaction – The ligation of synthesized C-terminal thioesters with N-terminal Cys-C99 was performed by mixing 1 mM synthesized C-terminal thioester and 0.5 mM Cys-C99 in 50 mM Tris-HCl, 100 mM glycine, pH 8.5, 0.2 % Triton X-100, and a suitable thiol additive (i.e., 20 mM 2-mercaptoethanesulfonic acid or 2% (vol/vol) thophenol). The ligation reaction was performed at 4◦C and was vortexed periodically. Efficient ligation reaction was also observed when performed in the presence of 3 M guanidine hydrochloride or 10% DMSO. The ligation products

were dialyzed against 50 mM Tris-HCL, pH 7.4, 0.2% Triton X-100 before being used in subsequent γ-secretase assays.

Results

Nicastrin and γ-substrates APPC99 or Notch100 associate directly and form a stoichiometric complex.

Both presenilin and nicastrin can bind to APP and Notch derived γ-secretase substrates. Whether nicastrin binds to substrates directly or indirectly and the stoichiometry of this physical association remained to be determined. To examine the biochemical attributes of this association in further detail, we first obtained large quantities of proteins by infecting Sf9 cells with baculoviruses expressing NicastrinV5/His as well as C100Flag either individually or in combination. NicastrinV5/His from Sf9 cells runs at ~ 120KDa on SDS-Page and is sensitive to PNGase-F and partially to Endo-H digestion (data not shown). Short carboxyl-terminal fragments of ~ 10 kDa in Sf9 cells infected with NicastrinV5/His were observed. These observations are consistent with previous studies where nicastrin is overexpressed. These short nicastrin derivatives are likely to be membrane bound and may contain the whole or at least a portion of the transmembrane region because they exist exclusively in membrane fractions and could only be extracted with detergents (Fig 4.1).

To determine whether nicastrin interacts with γ-secretase substrates directly, cell membranes from NicastrinV5/His, NicastrinV5/His plus C99Flag, or C99Flag alone were expressed in Sf9 cells. They were enriched using differential ultracentrifugation, washed with high salt buffer, and extracted with 1% Triton X-100. The membrane extracts were

subjected to co-immunoprecipitation with immobilized anti-FLAG M2 antibody, and the resultant co-immunoprecipitation products were eluted with Flag peptide and separated on SDS-PAGE gels. Analyses of coomassie stained SDS-PAGE gels indicate that NicastrinV5/His coprecipitated with C99Flag only in detergent extracts from Sf9 membrane where the two proteins were co-expressed. Western blotting analyses confirm that only full length NicastrinV5/His but not the membrane tethered C-terminal derivatives coprecipitated with C99Flag on immobilized anti-Flag M2 antibody, suggesting that the cytoplasmic and transmembrane regions of nicastrin are not critical for binding to C99. To determine the stoichiometry of the nicastrin and C99 complex, Flag peptide eluates from immobilized anti-Flag M2 antibody were subjected to affinity precipitation against NicastrinV5/His using immobilized Ni-NTA. Retention of C99Flag on immobilized Ni-NTA is specifically and stoichiometrically dependent on the presence of NicastrinV5/His. Densitometry analyses of the coomassie-blue stained bands corresponding to NicastirnV5/His and C99Flag show an approximate equal molar ratio (Fig 4.1).

The interaction between nicastrin and γ-secretase substrate C99 is highly specific. First, addition of Flag peptide to the membrane extracts from Sf9 cells co-expressing both proteins prevents purification of the NicastrinV5/His:C99Flag complex on immobilized anti-Flag M2 antibody. Similarly, addition of EDTA to the Flag peptide-eluted NicastrinV5/His:C99Flag complex prevented retention of the complex on immobilized Ni-NTA. Second, C99Flag purified with only full length nicastrin but not the membrane-

bound C-terminal derivatives. Third, under the conditions that preserved the association of nicastrin and C99 (Triton X-100 plus high salt washing), neither nicastrin nor C99

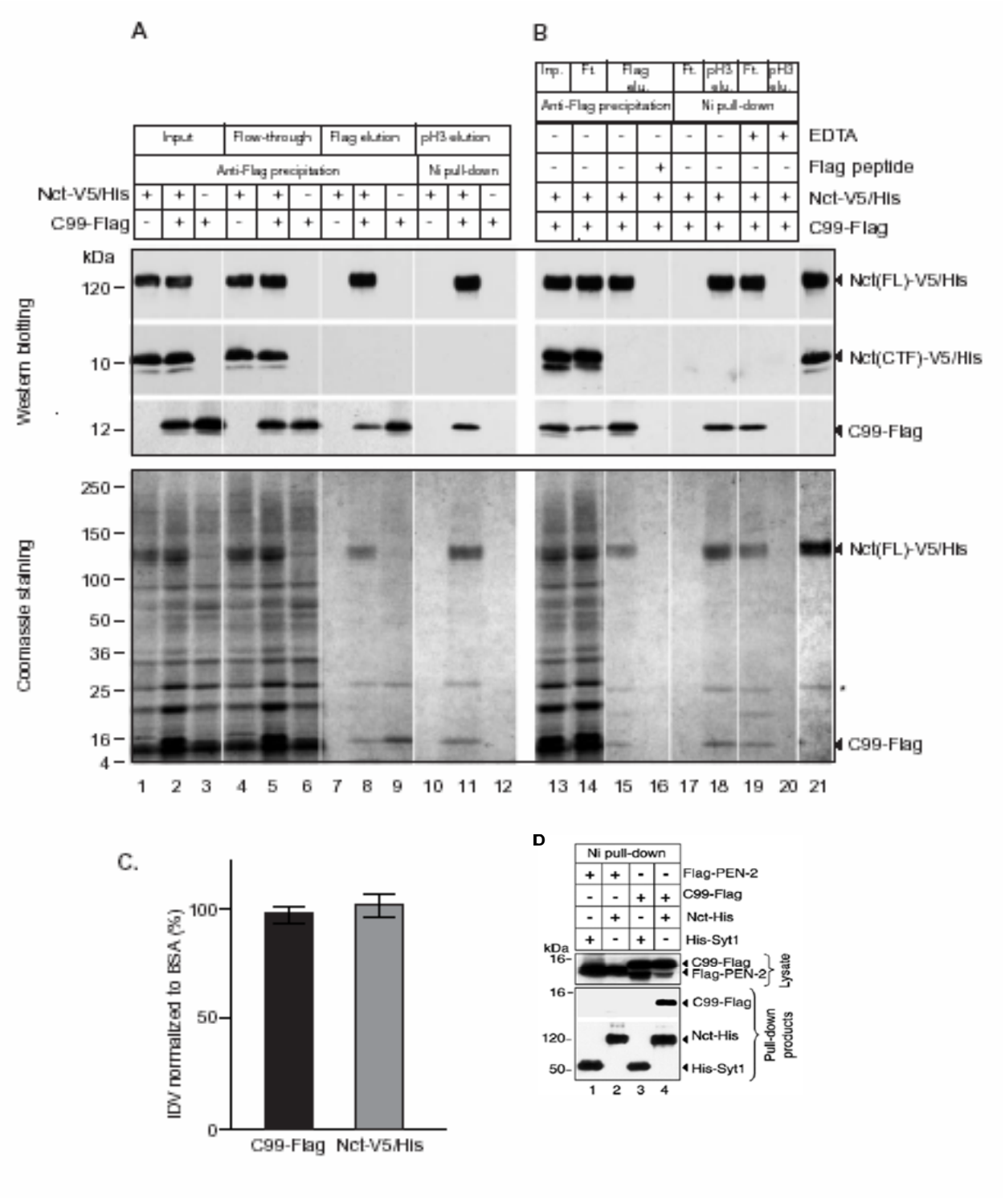


Figure 4.1. Stoichiometric interaction of full-length nicastrin and γ-secretase substrate C99. A) Triton X-100 extracts of Sf9 cell membranes expressing Nct-His, Nct-His plus C99-Flag, or C99-Flag were incubated with anti-Flag resin. A portion of the input (lanes 7-9) were analyzed on SDS-PAGE gels. The remaining Flag-peptide eluates were subjected to Ni-NTA pull down experiments (lanes 10-12). B) Triton X-100 extract of Sf9 cell membranes co-expressing Nct-His and C99-Flag was incubated with anti-Flag resin in the absence (-) or presence (+) of Flag peptide. A portion of the input (lane 13), flow-through (lane 14) and Flag peptide eluates (lane 15 & 16) were analyzed on SDS-PAGE gels. The remaining eluates were mixed with Ni-NTA in the absence (-) or presence (+) of 15 mM EDTA. The resultant flow-through (lane 17 & 19) and products eluted with glycine –HCL at pH 3.0 (lanes 18 & 20) were analyzed. Lane 21 shows Nct-His purified from Sf9 membrane on Ni-NTA agarose under stringent conditions. The SDS PAGE gels in 1A and 1B were probed with anti-His and anti-Flag antibodies (top three panels) or stained with coomassie blue (bottom panel). C) Products equivalent to lane 11 were concentrated, separated on SDS-PAGE gels, and stained with coomassie blue. The relative amounts of the coomassie-stained Nct-His and C99-Flag bands were quantified by densitometry and compared to a protein standard (BSA). The approximately equal molar ratio (average of 5 measurements: 0.943:1.023) was obtained after taking into consideration the molecular masses of C99 and nicastrin and their tags. IDV, integrated density value. D) Specific interaction of γ-secretase substrate C99-Flag with nicastrin

binds Pen-2 or several other membrane proteins such as synaptotagmin-1. Fourth, even though the starting materials for the aforementioned experiments are detergent extracts containing many membrane proteins, some of which are rather abundant, only nicastrin consistently and stoichiometrically co-precipitates with C99. We did however observe a 25 kDa endogenous protein that somewhat inconsistently co-elutes with the NicastrinV5/His:C99Flag complex. Taken together, these observations suggest that the association of nicastrin with C99 is highly specific, stoichiometric, and likely to be direct (Fig 4.1).

Nicastrin ectodomain interacts with γ-secretase substrate APPC99.

Considering the unique topology of nicastrin in the γ-secretase complex, as well as the absence of detectable interaction between C99 and nicastrin C-terminal derivates, we decided to examine whether the large and unique nicastrin ectodomain directly binds γ-secretase substrate C99. To do so, we generated a construct for a hybrid protein where the nicastrin ectodomain was fused with the IgG at the carboxy terminus. We purified Nicastrin(ECD)-IgG on protein-A agarose from conditioned media of HEK293 cells expressing the hybrid construct. High yields (>5 mg/L) were routinely obtained from Nicastrin(ECD)-IgG expressed in the mammalian cells and secreted to the conditioned media. Purified Nicastrin(ECD)-IgG and C99Flag were mixed and subjected to protein A agarose precipitations. The resultant precipitation products were examined for the two proteins. We observed that C99-Flag specifically co-precipitated with Nicastrin(ECD)-IgG but not the IgG fusion protein for nicastrin residues 1-50, APP residues 1-205, and dopamine receptor α2D1. Taken together, these data suggest that γ-secretase substrate C99 directly binds to the ectodomain of nicastrin. Interestingly, the interaction of Nicastrin(ECD)-IgG and C99Flag is disrupted in the presence of total rat brain membrane lysate, suggesting that the association of nicastrin ectodomain and γ-secretase substrate C99 is highly specific and subject to competition from molecules in total brain membrane extracts (Fig 4.2).

We next utilized a protein expression system that could readily yield large quantities of purified untagged nicastrin ectodomain, which would enable us to further analyze the physical and functional interaction of nicastrin and γ-secretase substrate without the potential interference of the IgG tag that usually exists in dimmerized form.

The ectodomain of nicastrin was expressed in Chinese hamster ovary (CHO) cells as a fusion protein with the human growth hormone (hGH), and octahistidine tag and a cleavage site for tobacco eth virus (TEV) protease. This fusion protein was produced at

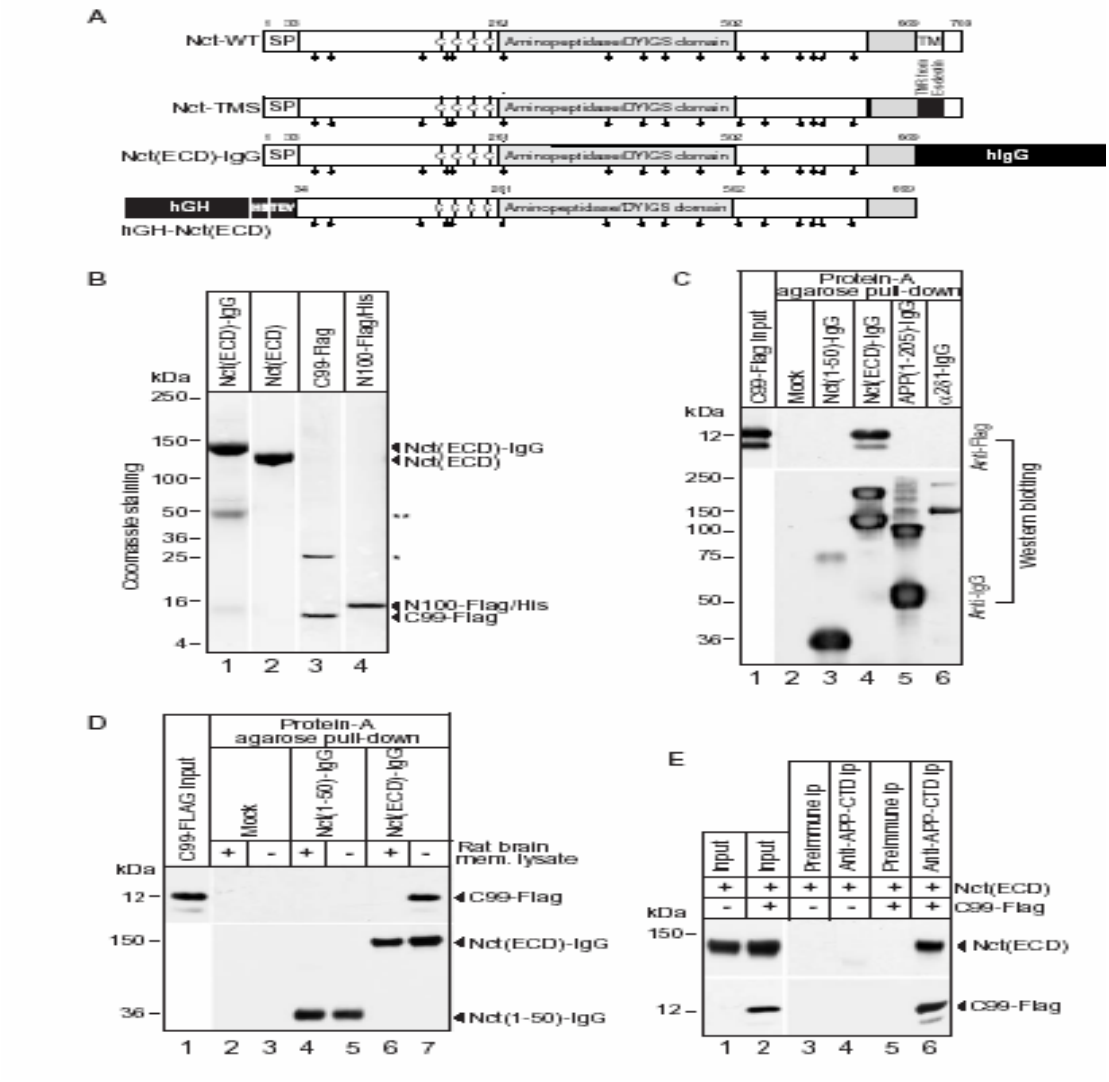


Figure 4.2. Nicastrin ectodomain interacts with γ-secretase substrate C99.
A) Schematic representation of nicastrin ectodomain fusion proteins. SP, signal peptide (residue 1-33): TM, transmembrane; Black box represents sequence from proteins foreign to Nct. Numbers indicated refer to the primary structure of human Nct. B) Tagged or untagged Nct(EC), C99, and N100 were purified and stained with coomassie blue. Mass spectrometry analysis shows that the 25 kDa band (*) is ADP/ATP translocase. The 50 kDa band (**) is probably a degredation product of Nct(ECD)-IgG as it reacts with an anti-Nct antibody. C) In mixture of C99-Flag and different IgG-fusion proteins, C99-Flagl co-precipitated with Nct(ECD)-IgG but not Nct(1-50)-IgG, APP(1-205)-IgG, or α2δ1-IgG on immobilized protein A. Dimerized IgG fusion proteins were visible due to precipitate with Nct(ECD)-IgG when total rat brain membrane lysate was added (+) to the mixture of the two proteins prior to protein A pull-down experiments. E) Nct(ECD) co-precipitates with C99-Flag in co-immunoprecipitation (IP) experiment using anti-APP-CTD but not preimmune serum. Nct(ECD) was not retained on anti-APP-CTD antibody or preimmune serum in the absence (-) of C99-Flag. The precipitation products in 2C, D, & E were probed with anti-Flag for C99-Flag, anti-human IgG for IgG-fusion proteins, and anti-Nct terminus for Nct(ECD).

(10 mg/L) and was initially purified by Ni-NTA immobilized metal affinity chromatography from conditioned media of the CHO cells. The fusion protein was then cleaved by TEV protease, and untagged nicastrin ectodomain was further purified by gel filtration and ReSource Q anion exchange chromatography to near homogeneity. Mixture of purified nicastrin ectodomain and C99Flag were subjected to co-immunoprecipitation with an antibody against the APP cytoplasmic domain. We observed specific co-precipitation of nicastrin ectodomain with C99Flag on immobilized anti-APP antibody but not on preimmune serum. We conclude that nicastrin ectodomain directly binds to γ-secretase substrate C99 (Fig 4.2).

Physical and functional interaction of nicastrin ectodomain with membrane tethered Notch (N100).

Nicastrin ectodomain binds the APP-derived γ-secretase substrate C99, we next investigated whether it could also physically interact with N100Flag/His, a carboxy terminal Flag/His-tagged, membrane-tethered Notch fragment that could be efficiently and specifically cleaved by γ-secretase. In experiments parallel to those described earlier, we observed that N100Flag/His co-purifies with Nicastrin-V5/His from Sf9 membrane extracts co-expressing these two proteins. We next mixed purified N100Flag/His and Nicastrin(ECD)-IgG and performed protein-A agarose precipitation experiments. We observed that N100Flag/His specifically co-precipitated with IgG fused nicastrin ectodomain but not IgG fused nicastrin residues 1-50, APP residues 1-205, and α2D1 (Fig 4.3).

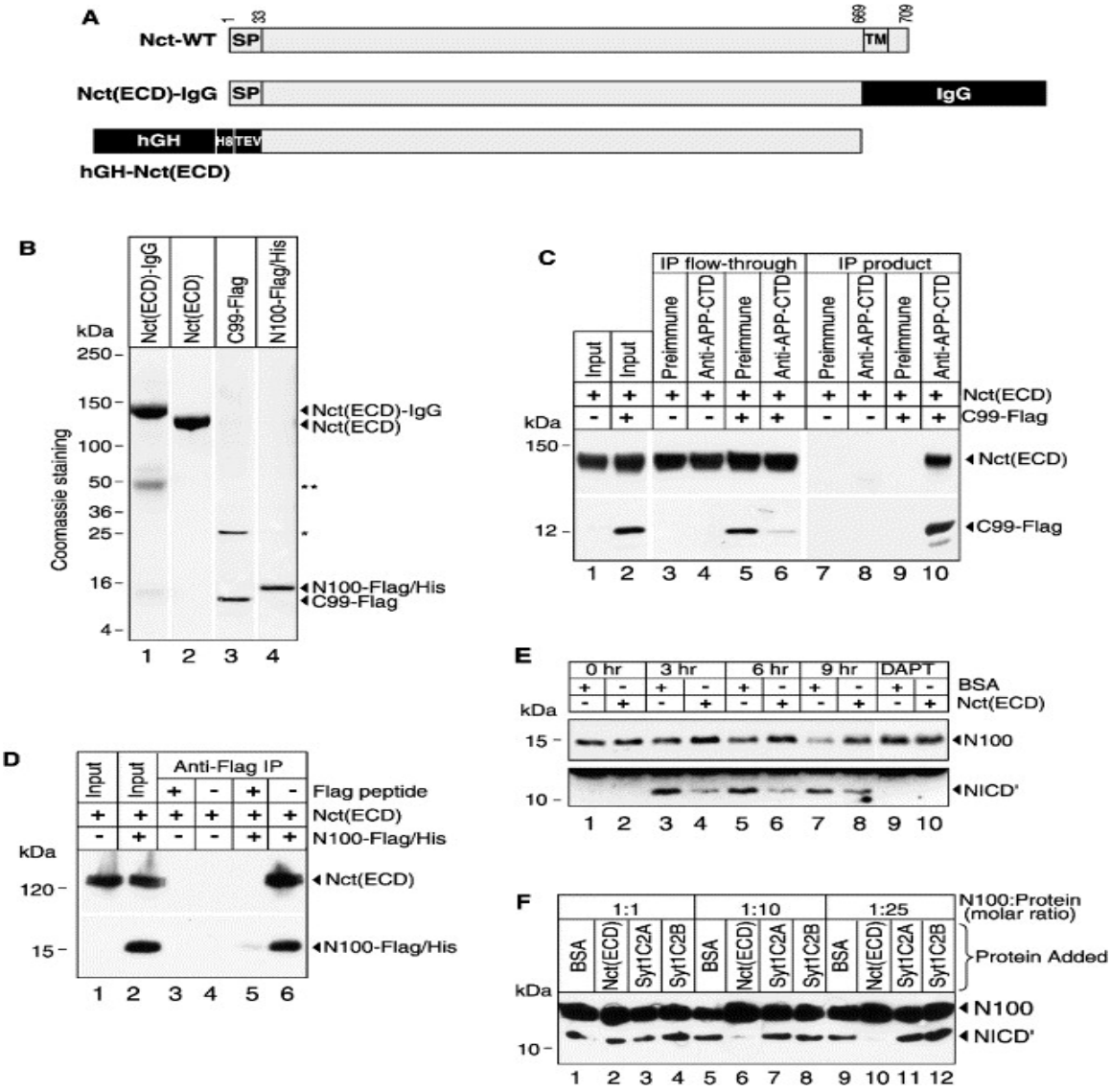


Figure 4.3. Nicastrin Ectodomain Physically and Functionally Interacts with APP- and Notch-Derived γ-Secretase Substrates A) Schematic representation of nicastrin-ectodomain fusion proteins. SP, signal peptide (residues 1–33); TM, transmembrane; black box represents sequence from proteins foreign to nicastrin. Numbers indicated refer to the primary structure of human nicastrin. B) Tagged or untagged Nct(ECD), C99, and N100 were purified and stained with Coomassie blue. The ~50 kDa band (**) is a degradation product of Nct(ECD)-IgG as it reacts with an anti-Nct antibody. C) Nct(ECD) coprecipitated with C99-Flag in IP experiments using anti-APP-CTD but not preimmune serum. Nct(ECD) was not retained on anti-APP-CTD or preimmune serum in the absence (−) of C99-Flag. D) Nct(ECD) coprecipitated with N100-Flag/His on anti-Flag resin only in a mixture of the two proteins. Addition (+) of Flag peptide into the mixture prevented the precipitation of either component. E) N100-Flag/His preincubated with either BSA or Nct(ECD) at 1:10 (N100:BSA or N100:Nct(ECD)) molar ratio was added to cell-free γ-secretase from HeLa cells and incubated at 37°C for 0, 3, 6, and 9 hr. In one sample set, γ-secretase inhibitor DAPT was added before incubating for 9 hr at 37°C. Longer incubation (e.g., 9 hr) at 37°C resulted in partial degradation of N100-Flag/His and NICD′-Flag/His, presumably by contaminating proteases in the crude cell-free γ-secretase preparation. The exposure times in the two panels differ due to the higher abundance of N100-Flag/His relative to NICD′-Flag/His. (F) N100-Flag/His preincubated with either BSA, Nct(ECD), Syt1 C2A, or Syt1 C2B at 1:1, 1:10, or 1:25 molar ratio was added to cell-free γ-secretase and incubated at 37°C for 3 hr. The samples in (C)–(F) were probed with anti-Nct N terminus for Nct(ECD) and anti-Flag for Flag-tagged C99, N100, and NICD′.

Similarly, untagged pure nicastrin ectodomain specifically co-precipitated with N100Flag/His on immobilized anti-Flag M2 antibody only from mixtures of the two proteins. We concluded that the ectodomain of nicastrin specifically and directly interacts with the Notch-derived γ-secretase substrate N100 (Fig 4.3).

To determine the functional significance of the interaction of nicastrin ectodomain and membrane tethered Notch, we studied the effect of nicastrin ectodomain on γ-secretase activity in a cell free assay. γ-Secretase was obtained from CHAPSO extracted HeLa cell membranes and purified N100Flag/His was used as γ-secretase substrate in this assay. Mixing N100Flag/His and purified nicastrin ectodomain before adding to the γ-secretase assay resulted in reduced generation of NICDFlag/His, a γ-secretase cleaved N100Flag/His product. In contrast, more γ-secretase substrate N100Flag/His accumulated in the reaction mixture where nicastrin ectodomain was added. The inhibitory effect on substrate cleavage correlates with the amounts of nicastrin ectodomain added to the reaction mixture (data not shown). We interpret these observations as that the ectodomain of nicastrin acts as an inhibitor by sequestering γ-secretase substrate N100Flag/His. Binding of nicastrin ectodomain with substrate may be a functionally important step that precedes proteolysis (Fig 4.3).

DYIGS/aminopeptidase domain but not the transmembrane domain of nicastrin is critical for γ-secretase substrate recognition.

To further investigate the structural and functional significance of nicastrin in γ-secretase substrate binding, we generated a chimeric nicastrin protein where the

transmembrane domain is swapped with another type-I glycoprotein E-selectin. The Nicastrin-TMS-V5/His mutant is transiently expressed in HEK293 cells stably expressing C99. Ni-NTA pull-down experiments revealed that the amount of C99 co-precipitated with Nicastrin-TMS-V5/His is comparable to that with V5/His-tagged wild type nicastrin (Nicastrin-WT-V5/His). In contrast, much less presenilin-1 NTF, and active species of prsenilin-1, co-precipitated with Nicastrin-TMS-v5/His when compared to Nicastrin-WT-V5/His. These data further support the conclusion that nicastrin ectodomain is required for γ-secretase substrate binding. They also suggest that nicastrin TMR is important for γ-secretase assembly but is not essential for substrate binding (Fig 4.4).

A nicastrin mutant that interacts with γ-secretase substrate but can not properly incorporate into the active γ-secretase complex should have a dominant negative like effect on γ-secretase activity. To test this, we established HEK293 cell lines stably expressing Nicastin-TMS-V5/His and Nicastrin-WT-V5/His. A well established and very sensitive γ-secretase assay to examine intramembrane cleavage of C99 was used. We observed time-dependent and expression dependent inhibition of γ-secretase activity in cells stably expressing Nicastin-TMS-V5/His when compared to Nicastirn-WT-V5/His. This inhibitory effect is highly significant but not drastic. This is consistent with earlier findings that γ-secretase assembly is tightly regulated by each of the four γ-secretase subunits and is difficult to overcome in mammalian cells. Taken together, we conclude that nicastrin ectodomain physically and functionally interact with γ-secretase substrate C99 (Fig 4.4).

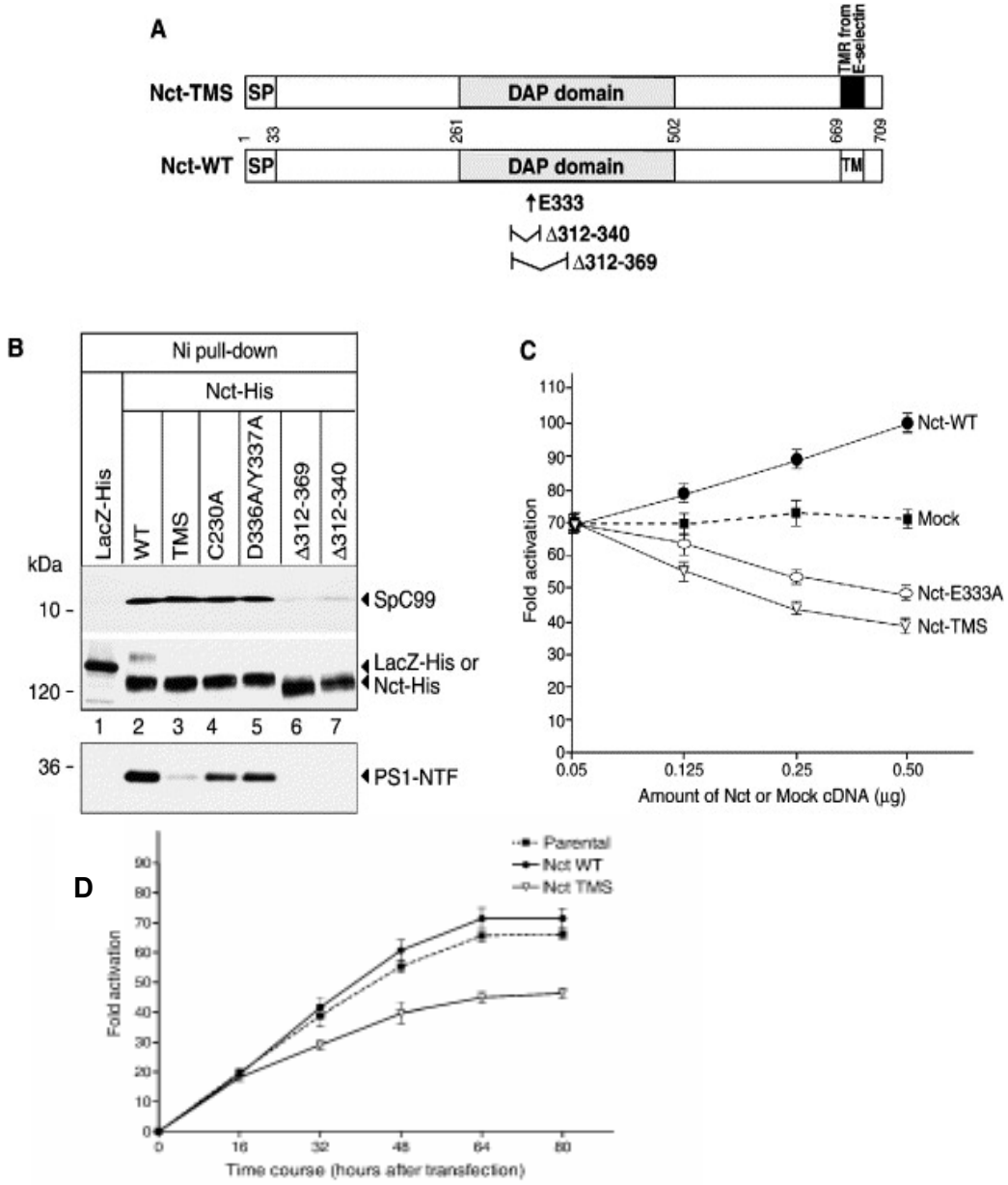


Figure 4.4. The Ectodomain but Not the TMR of Nicastrin Is Critical for γ-Secretase-Substrate Recognition A) Schematic representation of nicastrin proteins (Nct-wt, TMS, Δ312–340, and Δ312–369) and the position of Glu333. DAP domain, *D*YIGS *a*nd *p*eptidase homologous domain. B) HEK293 cells stably expressing SpC99 were transiently transfected with constructs encoding one of the following proteins: LacZ-His; His-tagged Nct-wt, TMS, C230A, D336A/Y337A, Δ312–369, or Δ312–340. After determining that similar amounts of SpC99, His-tagged proteins, and endogenous PS1 were expressed in each set of cells, we subjected equal amounts of Triton X-100 cell lysates to Ni-NTA pull-down in a high-salt buffer. The resultant products were probed with anti-APP-CTD or anti-His (top panels). In parallel experiments, CHAPSO lysates were subjected to Ni-NTA pull-down. The resultant products were investigated with anti-PS1-NTF (bottom panel). C,) HEK293 cells were transiently cotransfected with the indicated amounts of *Nct* cDNAs or empty vector (Mock), APP-GV or a plasmid containing only the Gal4 DNA binding domain, and β-galactosidase. Samples were assayed for luciferase reporter activity on 96-well plates. Data collected were normalized to the β-galactosidase activity prior to presenting the results as fold activation over the Gal4-transfected samples. Data are represented as mean ± SEM. D) Timecourse of APP-GV assay.

DYIGS/aminopeptidase (DAP) domain and residue Glu333 of Nicastrin is required for γ-secretase substrate recognition in vivo.

To further study the role of the nicastrin ectodomain in substrate recognition and to delineate the critical substrate binding site in the ectodomain of nicastrin at physiological levels, we generated *nicastrin* knockout mice and obtained fibroblasts from these mice and their littermates. As expected, neither immature (hypoglycosylated) nor mature (hyperglycosylated) nicastrin was detectable in $Nct^{-/-}$ fibroblasts. Presenilin endoproteolytic fragments were also not detectable in the *nicastrin*-deficient cells. In contrast, the β- and α-secretase-generated APP C-terminal fragments C99 and C83 were markedly increased. Membrane extracts from the $Nct^{-/-}$ cells were incapable of cleaving the Notch- and APP-derived γ-secretase substrates (N100 and C99) in cell-free assays (data not shown). APP-GV transactivation assay also showed no detectable γ-secretase activity. Transient expression of Nct-wt in the $Nct^{-/-}$ fibroblasts increased γ-secretase activity ~150-fold to a level comparable to that in the wild-type cells. Significantly, expression of Nct-TMS in *nicastrin*-deficient cells did not restore γ-secretase activity.

Having demonstrated that the $Nct^{-/-}$ fibroblasts were bona fide knockout cells that lack γ-secretase activity, we analyzed in these cells the structural and functional effects of several mutations in the conserved DAP domain of nicastrin. The DAP domain was of interest because primary-structure analysis revealed potential substrate binding residues in this region based on known aminopeptidase structures. In addition, an earlier study indicated that the DYIGS motif was critical for nicastrin function in intramembrane proteolysis (Yu, Nishimura et al. 2000) (Fig 4.5).

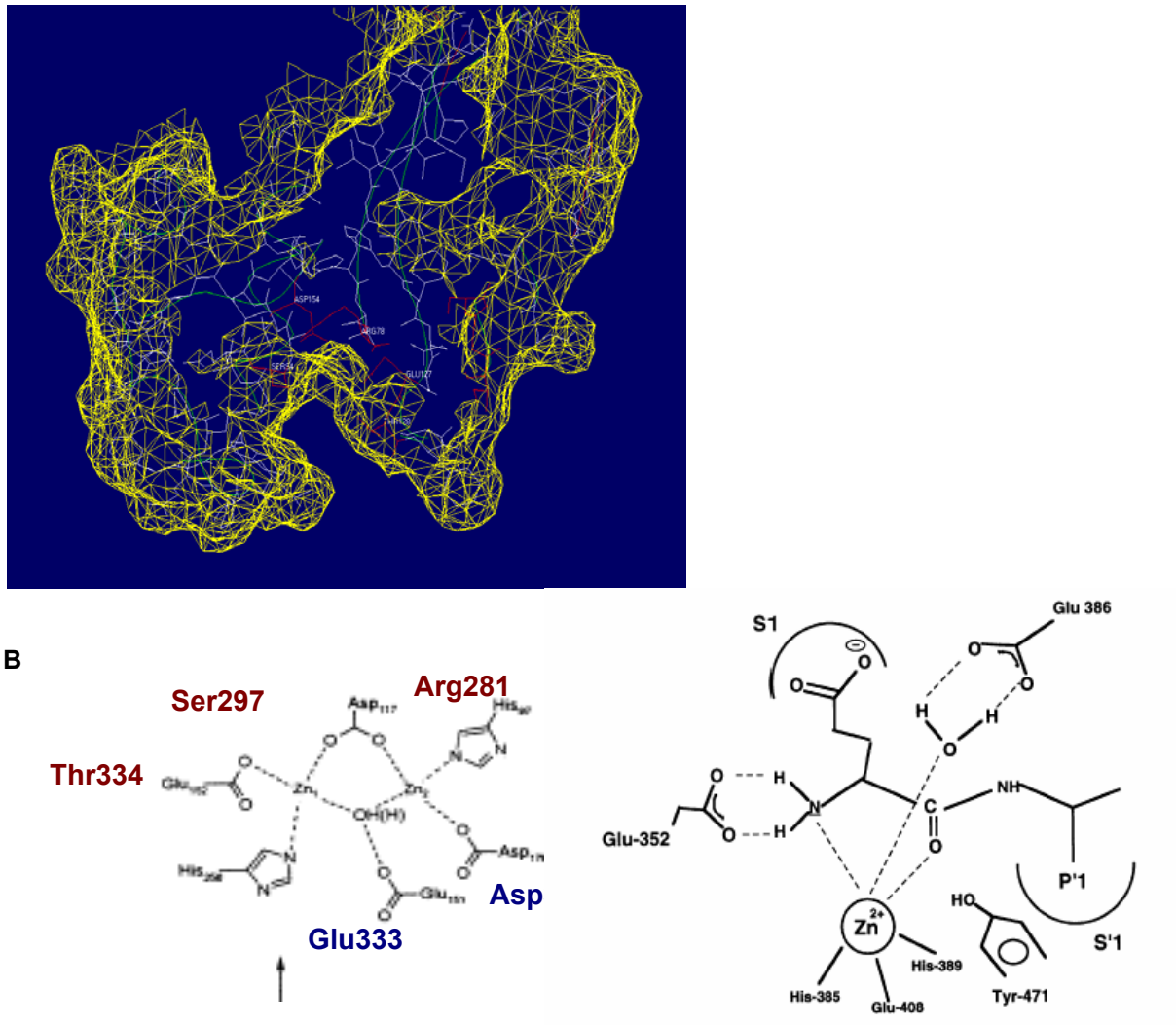


Figure 4.5. Modeling of DAP domain of nicastrin. A) Swiss PDG viewer NBS programming of 3D predictions based on homology to di-zinc aminopeptidases. B) Putative or predicted interaction site of N-terminal alpha amine of peptide substrate or inhibitor with the conserved glutamate 333 residue in nicastrin ectodomain.

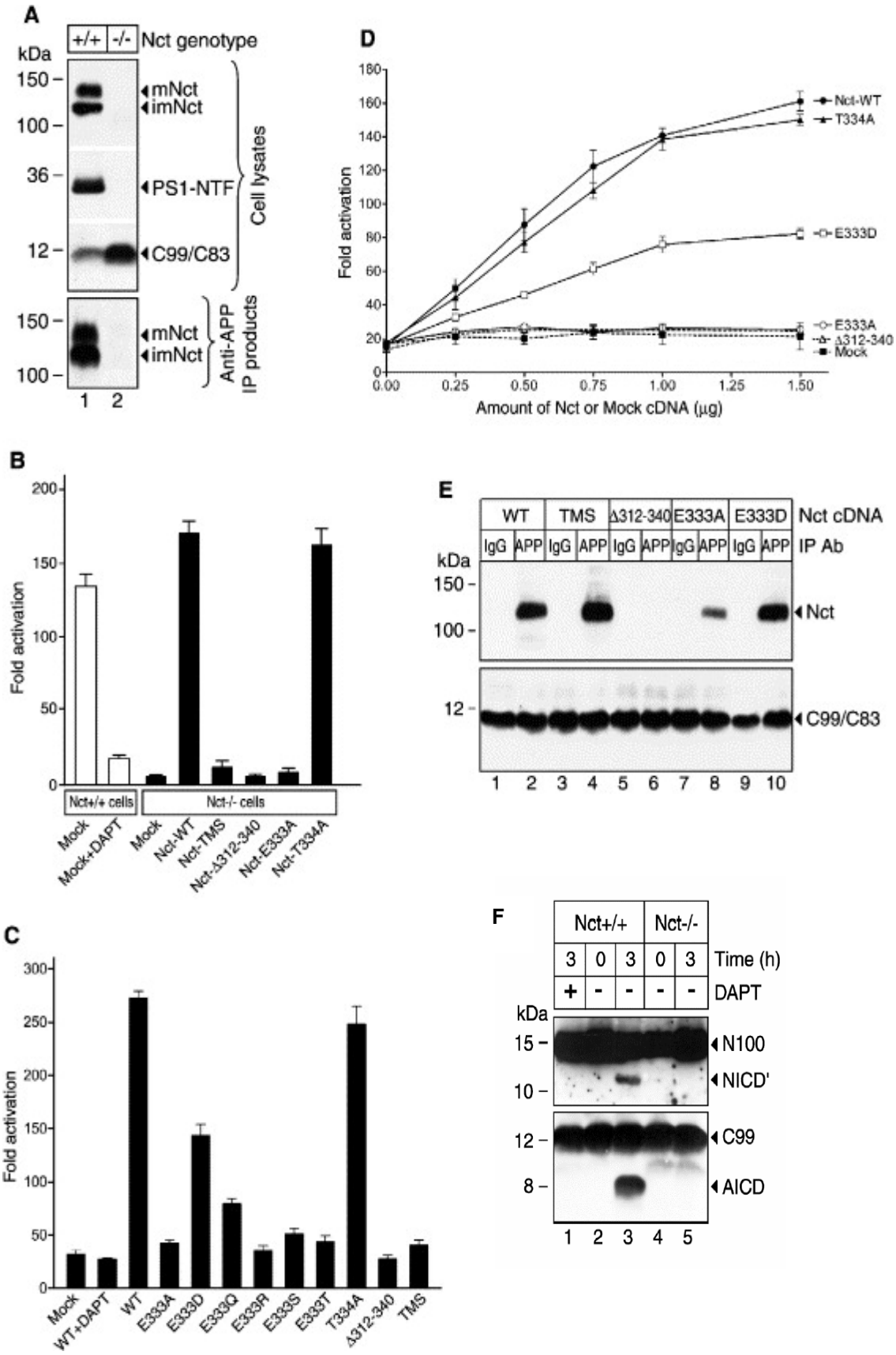

A
+/+
-/-
Nct genotype
kDa
150
100
36
12
mNct
imNct
PS1-NTF
C99/C83
Cell lysates
150
100
mNct
imNct
Anti-APP
IP products
1 2
B
Fold activation
200
150
100
50
0
Nct+/+ cells
Nct-/- cells
Mock
Mock+DAPT
Mock
Nct-WT
Nct-TMS
Nct-Δ312-340
Nct-E333A
Nct-T334A
C
Fold activation
300
250
200
150
100
50
0
Mock
WT+DAPT
WT
E333A
E333D
E333Q
E333R
E333S
E333T
T334A
Δ312-340
TMS
D
Fold activation
180
160
140
120
100
80
60
40
20
0
0.00
0.25
0.50
0.75
1.00
1.25
1.50
Amount of Nct or Mock cDNA (μg)
Nct-WT
T334A
E333D
E333A
Δ312-340
Mock
E
WT
TMS
Δ312-340
E333A
E333D
Nct cDNA
IgG
APP
IP Ab
kDa
150
100
Nct
12
C99/C83
1 2 3 4 5 6 7 8 9 10
F
Nct+/+
Nct-/-
3 0 3 0 3
Time (h)
+ - - - -
DAPT
kDa
15
10
N100
NICD'
12
8
C99
AICD
1 2 3 4 5

Figure 4.6. The DAP Domain of Nicastrin Is Required for γ-Secretase-Substrate Recognition In Vivo. A) Top three panels: Western blotting for Nct, PS1-NTF, and APP C-terminal fragments C99/C83 in cell lysates from embryonic fibroblasts of *nicastrin* knockout mice (−/−) and their wild-type littermates (+/+). Bottom panel: Western blotting for nicastrin in the anti-APP-CTD IP products from Triton X-100 extracts of wild-type and $Nct^{-/-}$ fibroblasts. No detectable nicastrin was coprecipitated with preimmune serum in parallel experiments (not shown). mNct, mature nicastrin; imNct, immature nicastrin. B) Wild-type and $Nct^{-/-}$ cells transfected with 0.5 μg/well of *Nct* cDNAs or empty vector (Mock) were analyzed for APP cleavage on 96-well plates using the APP-GV transactivation assay as in figure 4D. Data are represented as mean ± SEM. C) $Nct^{-/-}$ cells complemented with 0.5 μg/well *mock or Nct* cDNAs were analyzed with the C99-GV transactivation assay. Data are represented as mean ± SEM. D) $Nct^{-/-}$ cells transfected with the indicated amounts of cDNAs were analyzed with the C99-GV transactivation assay. Data are represented as mean ± SEM. E) $Nct^{-/-}$ fibroblasts complemented with the *Nct* cDNAs indicated on the top were subjected to overnight treatment with γ-secretase inhibitor DAPT. After confirming that each set of cells contained similar protein levels of C99/C83 (bottom panel) as well as nicastrin, Triton X-100 extracts were subjected to IP with anti-APP-CTD and control IgG. Samples in (A) and (E) were electrophoresed on SDS-polyacrylamide gels and probed with appropriate antibodies for Nct, PS1-NTF, and APP intracellular domain. F) In vitro γ-secretase assay from nicastrin knockout cell membranes and controls

APP-GV and C99-GV transactivation assays revealed that a deletion of 28 amino acids (residues 312–340) in the DAP domain of nicastrin abolished γ-secretase activity. Using known aminopeptidase structures as a model, we hypothesized that a conserved glutamate residue (Glu333) within this 28 amino acid region of nicastrin is positioned in an analogous substrate binding pocket. The equivalent glutamate residue in the active sites of many aminopeptidases is implicated in exopeptidase specificity via multiple interactions with the substrate, particularly the interaction of the carboxylate side chain of the active-site glutamate and the free N-terminal α-amino group of the substrate. Accordingly, we examined whether mutation of Glu333 to Ala affects APP processing. Like Nct-TMS and Δ312–340, E333A mutation abolished intramembrane cleavage of APP in the $Nct^{-/-}$ cells and inhibited γ-secretase activity in HEK293 cells. Similarly, E333R, E333S, and E333T mutations inactivated γ-secretase activity in *nicastrin*-deficient cells, whereas the conservative E333Q mutation preserved residual γ-secretase activity. In contrast, mutations of several other conserved residues in the DAP domain of nicastrin (e.g., T334A, T280A, S297A, and D360A) did not grossly affect γ-secretase cleavage of APP. To examine the role of the carboxylate side chain of Glu333 in mediating substrate interaction, we mutated nicastrin glutamate 333 to aspartate. If a similar molecular logic of substrate recognition applies to both aminopeptidase and nicastrin, the Nct-E333D mutation should not inactivate γ-secretase activity. Indeed, significant (albeit reduced) γ-secretase activity was observed in $Nct^{-/-}$ fibroblasts expressing Nct-E333D. These observations support the model that Glu333 and the DAP domain of nicastrin are important in substrate recognition (Fig 4.6).

We next examined whether nicastrin physically interacts with γ-secretase substrates in vivo and whether this interaction is affected by mutations in the DAP domain. In our complementation experiments in *Nct*$^{-/-}$ cells, the steady-state level of exogenous nicastrin and the restored γ-secretase activity were comparable to those in the wild-type cells. This reflects the highly restricted nature of γ-secretase assembly and allows analysis of the substrate binding properties of nicastrin mutants at physiological protein levels. After confirming that endogenous nicastrin associates with C99/C83 in native wild-type cells but not in *nicastrin*-deficient cells , we performed IP experiments under stringent conditions using high-salt Triton X-100 extracts from *nicastrin* knockout cell membranes expressing similar levels of Nct-wt, TMS, Δ312–340, E333A, and E333D. Because the level of C99/C83 is lower in the *nicastrin* knockout cells complemented with Nct-wt than in those with inactive nicastrin mutants (e.g., TMS, Δ312–340, and E333A), each set of cells was treated with γ-secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester) prior to detergent extraction so that the C99/C83 amount was comparable in each sample. While binding of nicastrin and C99/C83 was not grossly affected by the TMS or E333D mutation, significantly reduced amounts of Nct-E333A and Δ312–340 coprecipitated with C99/C83. The observation that C99/C83 binding was not significantly affected by E333D mutation is consistent with our earlier functional data and supports the model that carboxylate side chain of Glu333 is involved in substrate binding. We conclude that the nicastrin ectodomain, particularly the Glu333 and its nearby residues in the DAP domain, is required for substrate binding (Fig 4.6).

Reconstitution of recombinant γ-secretases and analysis of the effects of mutation Glu333Ala on presenilin endoproteolysis and γ-secretase activity.

To determine the functional role of the nicastrin Glu333 site in γ-secretase activity, we reconstituted γ-secretase in Sf9 cells using baculoviruses expressing nicastrin (either wild-type or E333A), full-length presenilin-1 (wild-type or D257A), APH-1, and PEN-2. As expected, catalytic site mutation D257A inhibited generation of presenilin-1 endoproteolytic fragment NTF. Presenilin-1 NTF was efficiently generated in the reconstituted γ-secretase where nicastrin wild-type or E333A mutant was used. This observation suggests that Glu333 is not essential for presenilin endoproteolysis, which likely requires fully assembled complex of presenilin, nicastrin, APH-1, and PEN-2. Presenilin endoproteolytic fragments could only be generated when all four proteins are co-expressed. We next tested if γ-secretase cleavage of type-I membrane protein with short N-terminus is affected by nicastrin E333A mutation in reconstituted recombinant γ-secretase from Sf9 cells. As expected, presenilin D257A mutation or γ-secretase inhibitors (DAPT, inhibitor IV, and L685,458) prevented cleavage of N100Flag/His by the reconstituted γ-secretases. Remarkably, γ-Secretase cleavage of N100Flag/His and generation of NICD' were significantly inhibited when reconstituted γ-secretase containing nicastrin E333A mutation was used. Not unexpectedly, intramembrane cleavage of C99Flag was also inhibited when recombinant γ-secretase containing the presenilin D257A mutation was used. Importantly, mutant γ-secretase containing nicastrin E333A produced much less AICD. Thus, our investigations show that nicastrin E333A mutation preferentially affects γ-secretase cleavage of type-I membrane proteins

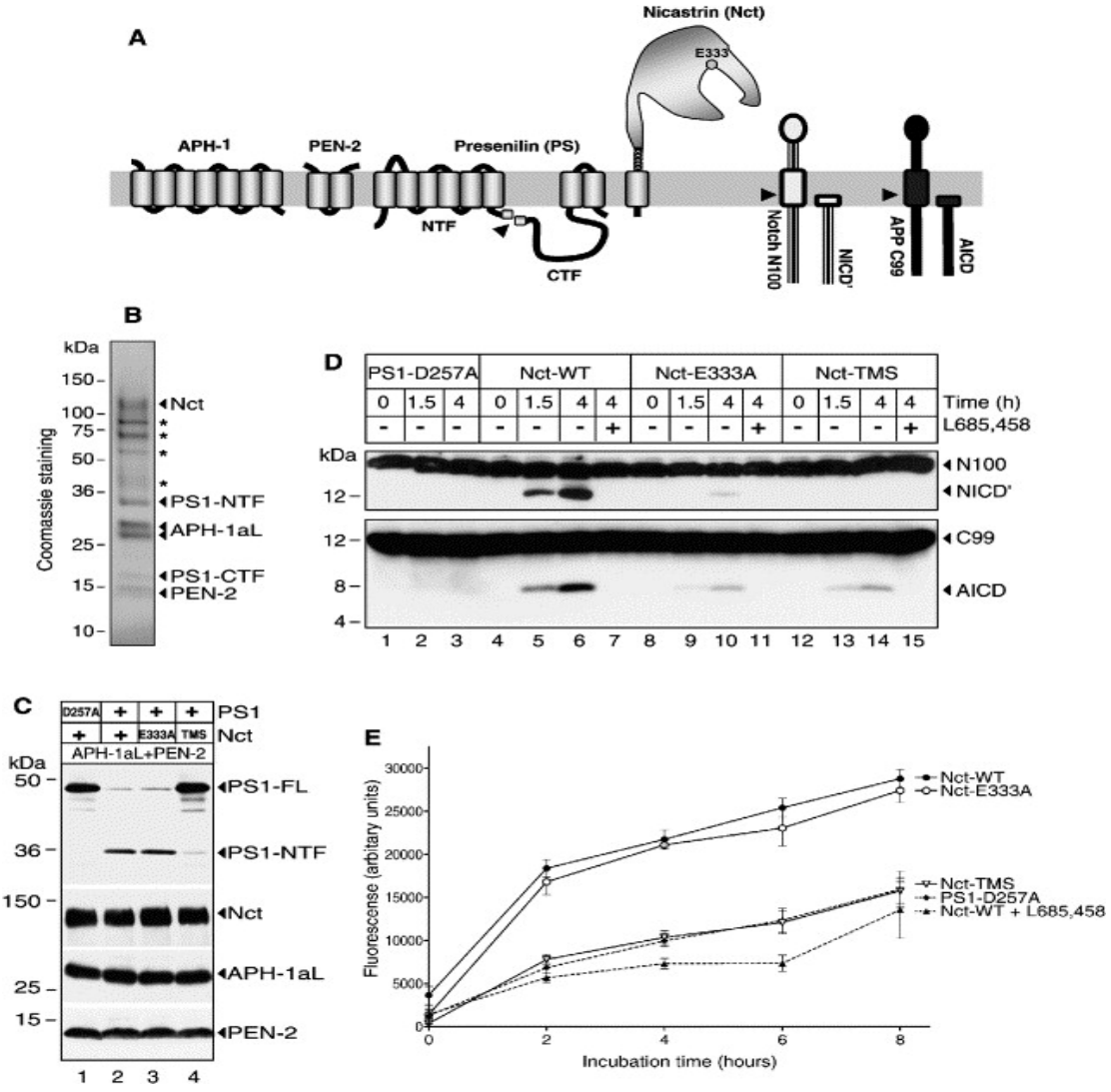


Figure 4.7. Nicastrin DAP Domain Is Required for Substrate Recognition In Vitro. A) Schematic representation of γ-secretase subunits and substrates derived from APP and Notch. Arrow points to the site of presenilin endoproteolysis or γ-secretase cleavage. AICD, APP intracellular domain; NICD′, a truncated Notch intracellular domain. B) γ-secretase purified from Sf9 membranes coexpressing Nct, PS1, APH-1aL, and PEN-2 was separated on SDS-polyacrylamide gel and stained with Coomassie blue. The identities of several additional proteins (*) that copurified with γ-secretase activity are currently under investigation. C) Membrane-protein complexes isolated from Sf9 cells coinfected with baculoviruses harboring PS1 (wild-type [+] or D257A), Nct (wild-type [+], E333A, or TMS), APH-1aL, and PEN-2 were analyzed by Western blotting for full-length and endoproteolytic fragments of PS1 as well as the other γ-secretase components as indicated. D) Recombinant γ-secretase preparations from (C) were subjected to incubation with N100-Flag/His (top panel) or C99-Flag (bottom panel) at 37°C for 0, 1.5, and 4 hr, with (+) or without (−) γ-secretase inhibitor L685,458. Samples were electrophoresed on SDS-polyacrylamide gels and probed with anti-Flag or anti-APP-CTD. E) γ-secretases from (C) were incubated with 8 μM intramolecularly quenched fluorogenic peptide probe Nma-GGVVIATVK(Dnp)*rrr*-NH_2 at 37°C. Fluorescence was measured at the time points indicated. Background fluorescence of the peptide probe alone was subtracted from all readings. Data are

whereas generation of steady-state presenilin endoproteolytic fragments by "presenilinase" activity is not significantly affected (Fig 4.7).

One caveat to this conclusion is that nicastrin E333A mutation could dynamically affect γ-secretase assembly and presenilinase activity. For example, nicastrin E333A mutation could reduce the binding affinity to γ-secretase substrates which although may not grossly prohibit but may still quantitatively change assembly of the γ-secretase complex and the presenilinase activity. Nevertheless, our data indicate that Glu333 in the DYIGS/aminopeptidase domain of nicastrin is responsible for binding to the amino-terminal parts of γ-secretase substrates and that alterations of this binding affects intramembrane cleavage of type-I membrane proteins (Fig 4.7).

The extracellular stubs of γ-secretase substrates are responsible for binding to nicastrin ectodomain.

Our finding that γ-secretase substrate binding site resides in the ectodomain, not the transmembrane domain or cytoplasmic domain, of nicastrin imply that the N-terminal short extracellular stub of γ-secretase substrate could participate in the interaction. We reasoned that blockage of the N-terminus, but not the C-terminus, of γ-secretase substrate would protect the substrate form being recruited by nicastrin to the γ-secretase complex form intramembrane cleavage. To test this hypothesis, we generated a baculovirus construct that expresses an N-terminal Flag tagged C99 (nFlag-C99) where the first seven residues (DAEFRHD) following the β-secretase cleavage site were replaced by the Flag epitope (DYKDDDDK). We co-expressed NicastrinV5/His with either nFlag-C99 or

C99Flag (C-terminal Flag-tagged C100) in Sf9 cells. In co-immunoprecipitation experiments, using an antibody against the cytoplasmic domain of APP, NicastrinV5/His efficiently co-precipitates with either n-Flag-C99 or the C99-Flag. Similarly, both nFlag-C99 and C99Flag co-precipitate with NicastinV5/His in Ni-NTA pull down experiments. Thus, like the C-teriminal Flag tagged C99, the N-terminal Flag tagged C99 could form a complex with nicastrin. Remarkably, although both nFlag-C99 and C99Flag were effectively retained on immobilized anti-Flag M2 antibody, nicastrin only readily and specifically coprecipitates with C99Flag but not nFlag-C99 in co-immunoprecipitation experiments using anti-Flag M2 antibody. The most plausible explanation for this observation is that the anti-Flag M2 antibody blocks access of the N-terminus of nFlag-C99 to the binding pocket in the ectodomain of nicastrin (Fig 4.8).

To test the functional implication of blockage of the N-terminus of γ-secretase substrate, we pre-incubated the N- and C-terminal Flag-tagged C99 protein with anti-Flag M2 antibody before using them as substrates for recombinant γ-secretase purified form Sf9 cells. As shown, addition of increased amounts of anti-Flag M2 antibody resulted in potent inhibition of γ-secretase cleavage of nFlag-C99 to generate AICD. In contrast, C99Flag cleavage was not significantly affected by addition of anti-Flag M2 antibody in parallel experiments. Addition of control antibody (normal IgG) to either the nFlag-C99 or C99Flag also has no effect on the cleavage of these substrates by recombinant γ-secretase under the same conditions. The following observations coupled with those described earlier, suggest that the extracellular N-terminal portion of γ-secretase substrate is the site recognized by the nicastrin ectodomain (Fig 4.8).

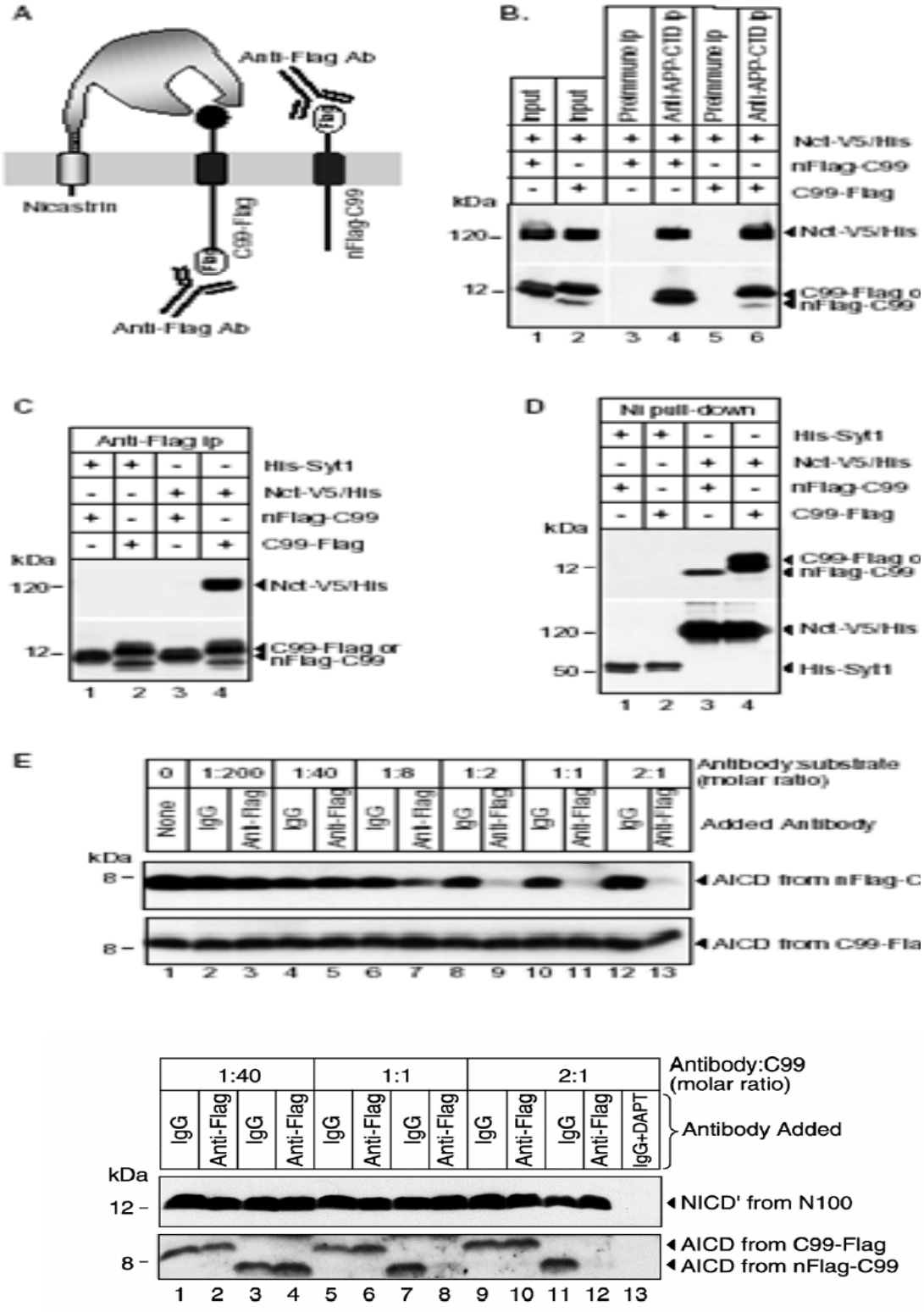

A
Anti-Flag Ab
Nicastrin
C99-Flag
nFlag-C99
Anti-Flag Ab
B.
Input
Input
Preimmune ip
Anti-APP-CTD ip
Preimmune ip
Anti-APP-CTD ip
Nct-V5/His
nFlag-C99
C99-Flag
kDa
120–
12–
Nct-V5/His
C99-Flag or
nFlag-C99
1 2 3 4 5 6
C
Anti-Flag ip
His-Syt1
Nct-V5/His
nFlag-C99
C99-Flag
kDa
120–
12–
Nct-V5/His
C99-Flag or
nFlag-C99
1 2 3 4
D
Ni pull-down
His-Syt1
Nct-V5/His
nFlag-C99
C99-Flag
kDa
12–
120–
50–
C99-Flag or
nFlag-C99
Nct-V5/His
His-Syt1
1 2 3 4
E
0
1:200
1:40
1:8
1:2
1:1
2:1
Antibody:substrate (molar ratio)
None
IgG
Anti-Flag
Added Antibody
kDa
8–
AICD from nFlag-C
8–
AICD from C99-Fla
1 2 3 4 5 6 7 8 9 10 11 12 13
1:40
1:1
2:1
Antibody:C99 (molar ratio)
IgG
Anti-Flag
IgG+DAPT
Antibody Added
kDa
12–
NICD' from N100
8–
AICD from C99-Flag
AICD from nFlag-C99
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.8. **Antibody-Mediated Blocking of the N terminus of γ-Secretase Substrate Prevents Its Access to the Nicastrin Ectodomain.** A) Schematic representation of experimental design. Blocking the N terminus of nFlag-C99 by anti-Flag should prevent access to the binding site in nicastrin ectodomain and inhibit cleavage by γ-secretase. Blocking the C terminus of C99-Flag by anti-Flag should not grossly affect access to the binding site. Unblocked C99 or N100 should be able to access the substrate binding pocket in nicastrin. B) Membrane-protein extracts from Sf9 cells infected with baculoviruses expressing Nct-His plus either N- or C-terminally Flag-tagged C99 were subjected to IP with anti-APP-CTD or with preimmune serum. C) Membrane extracts from Sf9 cells expressing His-Syt1 or Nct-His plus either N- or C-terminally Flag-tagged C99 were subjected to Ni-NTA pull-down. D) Membrane extracts from (C) were subjected to anti-Flag IP. The resultant products in (B)–(D) were probed with anti-His for His-tagged nicastrin and Syt1 proteins and anti-Flag for Flag-tagged C99 proteins. E) C99-Flag or nFlag-C99 preincubated with either normal mouse IgG or anti-Flag at the indicated molar ratio was added to purified recombinant γ-secretase from Sf9 cells and incubated at 37°C for 4 hr. AICD generated from C99-Flag or nFlag-C99 was investigated with anti-APP-CTD. F) Specific inhibition of cleavage of nFlag-C99 but not N100 with anti-Flag antibody. Equal amount of N100-Flag/His was mixed with the same amount of either C99-Flag (lanes 1-2, 5-6, & 9-10) or nFlag-C99 (lanes 3-4, 7-8, & 11-13). The mixtures of N100 and C99 (N100-Flag/His plus C99-Flag or N100-Flag/his pusl nFlag-C99) were pre-incubated with either normal mouse IgG or anti-Flag antibody at the indicated molar ratio, combined with γ-secretase, and incubated at 37 degree for 4 hours. NICD' generated from N100 was investigates with anti-cleaved Notch-1 (Val1744) antibody, which recognizes the N-terminus of the intracellular domain after Notch is cleaved between Gly1743 and Val1744. AICD generated from C99-Flag or nFlag-C99 was investigated with anti-APP-CTD antibody. Generation of NICD' and AICD was inhibited by γ-secretase inhibitor DAPT (lane 13). Blocking the N-terminus of C99 only affects the initial recognition of C99, an unblocked substrate such as N100 is not affected in its recognition and subsequent cleavage by γ-secretase in the same reaction. This experiment shows that γ-secretase cleavage of neither N100 nor C99-Flag was significantly affected by pre-incubating the mixture of N100 and C99-Flag with anti-Flag antibody. In contrast, cleavage of nFlag-C99, but not N100, was quantitatively blocked with the mixture of N100 and nFlag-C99 was pre-incubated with anti-Flag at an antibody to substrate molar ratio of 1:1 or 2:1. The result confirms that the N-terminal portion of type-1 transmembrane polypeptides is a primary γ-secretase recognition site.

Chemical blocking of the free N terminus inhibits substrate cleavage by γ-secretase.

We reasoned that the free N-terminal α-amino group of substrates may constitute the recognition site that is disrupted upon changes in the carboxylate group of Glu333 in the DAP domain of nicastrin. Thus, chemically blocking the free α-amino group of C99 may prevent it from being cleaved. Chemoselective modifications to the free α-amino group of C99 were initiated by employing a proteolytic cleavage approach to generate a C99-Flag-derived substrate, Cys-C99. The N-terminal cysteine of Cys-C99 was ligated to thioester derivative (dipeptide Gly-Gly thioester with or without N-terminal fluorescein or biotin-LC) using native chemical ligation. The resultant product (fluorescein-Gly-Gly-Cys-C99 or biotin-LC-Gly-Gly-Cys-C99) harbors fluorescein or biotin-LC at the N terminus. γ-secretase cleavage of the fluorescein- or biotin-LC-labeled C99 was inhibited when compared to cleavage of C99 substrates with a free α-amino group: C99-Flag, Cys-C99, or Gly-Gly-Cys-C99, a ligation product of Gly-Gly-thioester and Cys-C99 (Fig 4.9, 4.10, 4.11).

In addition, we compared γ-secretase cleavage of N-formylated C99-Flag (fC99) and C99-Flag harboring a free α-amino group. fC99 was expressed in and purified from *E. coli* AG100A cells in the presence of actinonin. Bacterial protein synthesis initiates with formyl methionine followed by posttranslational removal of the formyl group by peptide deformylase (PDF). The PDF inhibitor actinonin blocks the deformylation process, and thus the resultant proteins retain an uncharged formyl group at the N terminus. Remarkably, cleavage of fC99 bearing this small modification (three atoms) was inhibited as compared to C99 containing a free α-amino group.

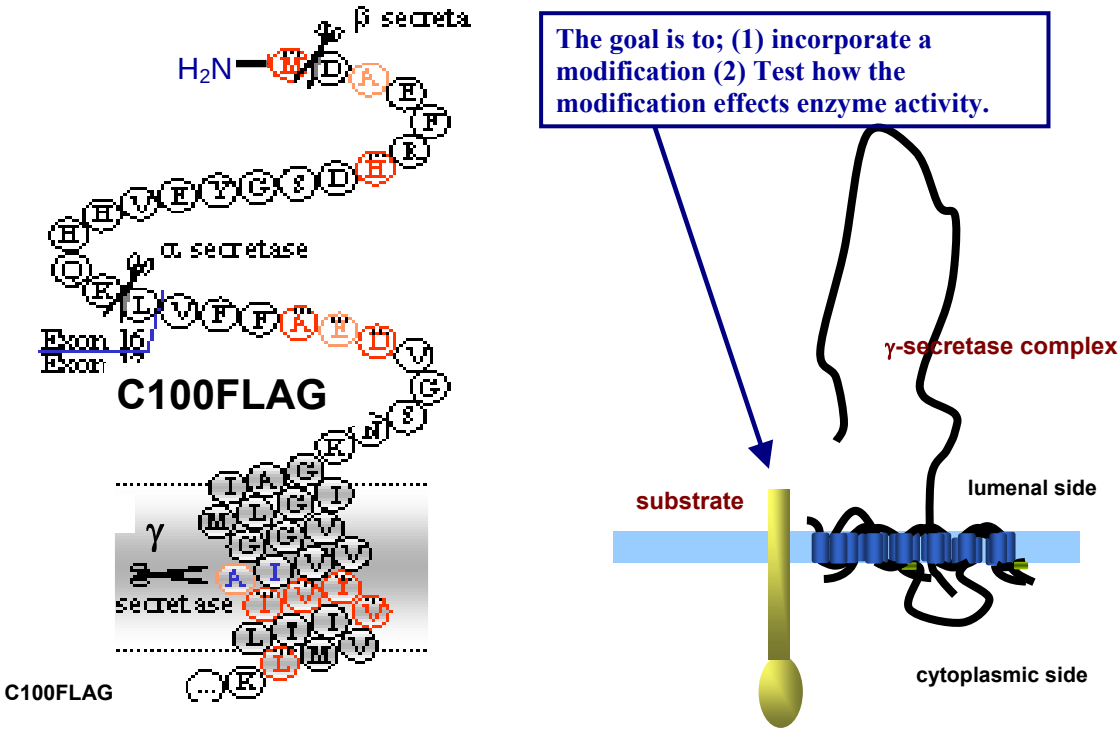


Figure 4.9. Goal was to develop new methods or take advantage of existing protocols to chemoselectively modify the alpha amino group of group I transmembrane protein. Here the moiety of interest is part of the backbone of the protein sequence. Unlike side chain residues that may be site directed mutated and tested in a functional assay, directed mutation of the alpha amine is not possible. The charged alpha amino group may be altered either using transamination or acetylation reactions, however these modification may also alter ε lysine side chain amines and thus are non-specific. Furthermore, many of these chemical reactions are inefficient in the presence of detergent thus limiting the chemistry under conditions that keep these hydrophobic membrane proteins from precipitating out of solution. We tested three approaches: (1) in vitro coupled Ecoli S30 tranlation/transcription to introduce a modified initiating met tRNA (2) native chemical ligaiton (3) expressing N-formylated proteins in bacterial strain treated with PDF inhibitor.

Strategy of Native Chemical Ligation

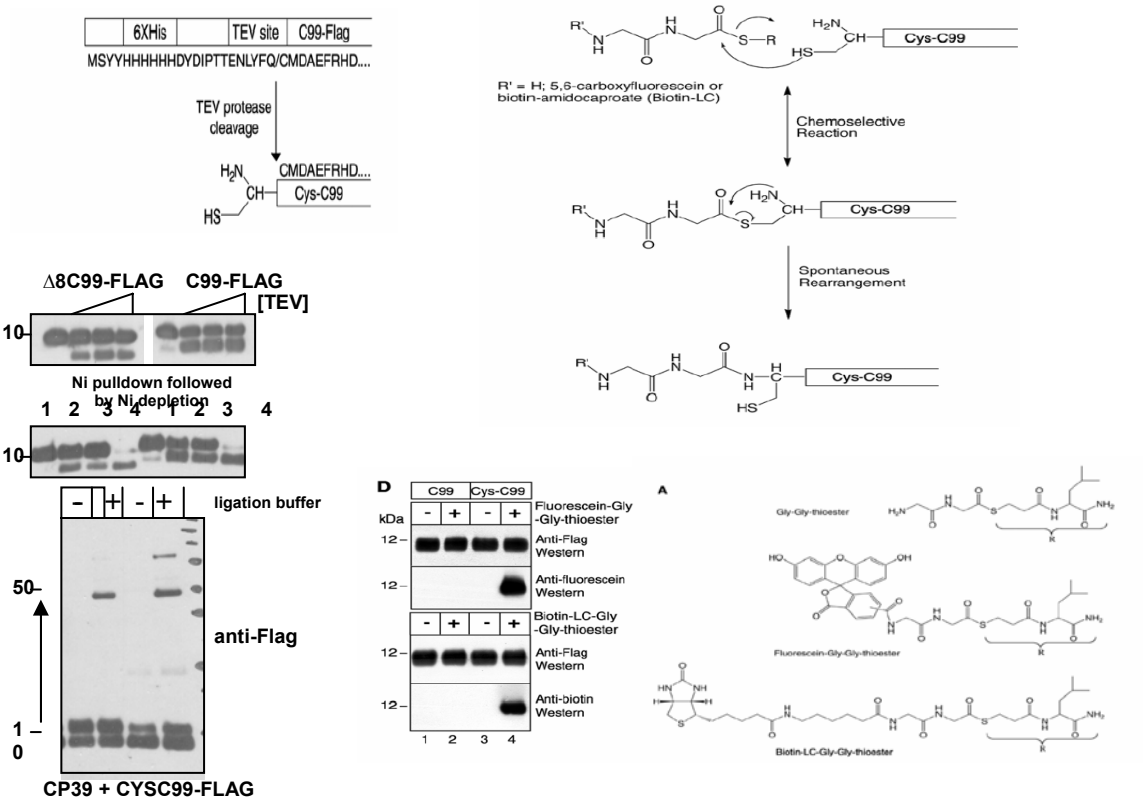
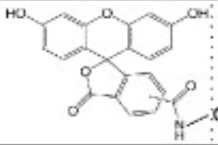
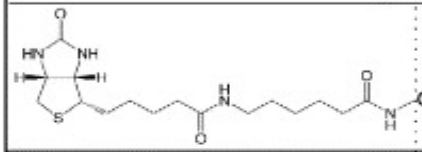
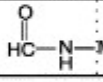
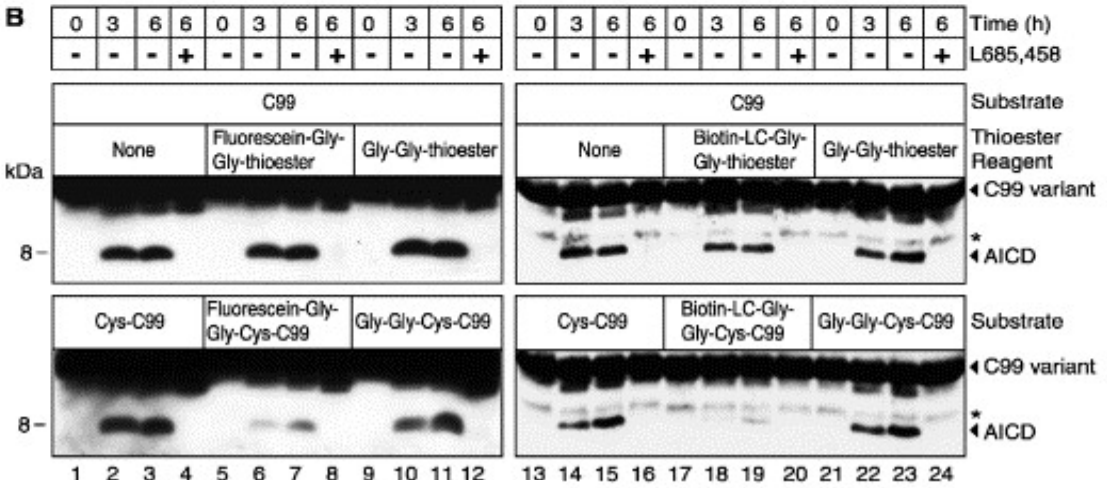


Figure 4.10. Native Chemical ligation approach to modifying N-terminal alpha amine. A technique used to coupling two short peptides to form a longer one due to the limitations in solid phase peptide synthesis. Of the two peptides, one must contain a C-terminal thioester and the second must contain an N-terminal cysteine. The N-terminal cysteine was designed into our immediate γ-secretase substrates using a tev site modification cleavage approach. N-terminal cysteine was confirmed by protein sequencing as well as coupling to a 39kDa carrier protein available from NEB. This process was developed by Phillip Dawson and Stephen Kent at The Scripps Research Institute in 1984, about the same time that inteins were discovered, which is nature's analogous reaction. Native chemical ligation is a two step reaction. The products result in an irreversible peptide bond formation. No protective groups are required for this reaction. This reaction occurs in water at physiological pHs, and internal cysteins remain undisturbed. Such chemical conditions were ideal to pusue this approach in our goal to modify the N-terminal alpha amine of membrane proteins we were interested in.

A Summary of C99 variants used in this study

Amino-terminal group	First five amino acids of C99 variants		γ-Secretase cleavage
H_2N—	Met-Asp-Ala-Glu-Phe···	*(C99)*	+
H_2N—	Met-Asp-Tyr-Lys-Asp···	*(nFlag-C99)*	+
H_2N—	Cys-Met-Asp-Ala-Glu···	*(Cys-C99)*	+
H_2N—	Gly-Gly-Cys-Met-Asp···	*(Gly-Gly-Cys-C99)*	+
	Gly-Gly-Cys-Met-Asp···	*(Fluorescein-Gly-Gly-Cys-C99)*	–
	Gly-Gly-Cys-Met-Asp···	*(Biotin-LC-Gly-Gly-Cys-C99)*	–
	Met-Asp-Ala-Glu-Phe···	*(fC99)*	–



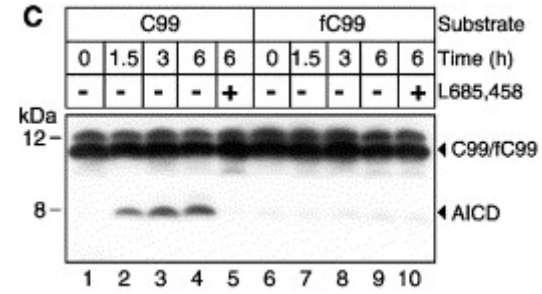


Figure 4.11. Chemical Blocking of the Free N Terminus Inhibits Substrate Cleavage by γ-Secretase. A) Summary of C99 variants showing the N-terminal chemical groups and their effects on γ-secretase cleavage. All C99 proteins were Flag tagged at the C termini except nFlag-C99, which has an N-terminal Flag tag. fC99, N-formylated C99-Flag; +, efficient cleavage; −, impaired cleavage. B) Cys-C99 was subjected to native chemical ligation in the presence or absence of the thioester derivatives. C99-Flag was treated with the thioesters under the same conditions (top panel). The resultant C99 variants were assayed for γ-secretase cleavage. Cleavage of the remaining unligated substrates may partly contribute to the residual AICD in lanes 6–7 and 18–19 of the bottom panel. Asterisk indicates a nonspecific protein insensitive to L685,458. C) Regular and N-formylated C99 were purified from bacterial cells in the absence and presence of actinonin. These substrates were subjected to γ-secretase-cleavage assays. D) Proposed role of nicastrin in γ-secretase-mediated RIP. γ-secretase is a high-molecular-weight complex composed of at least four membrane proteins: presenilin, nicastrin, APH-1, and PEN-2. γ-secretase substrates typically consist of type I membrane proteins with small extracellular stubs resulting from ectodomain shedding of the precursor proteins. The current study shows that nicastrin via its large ectodomain acts as a receptor for γ-secretase substrates by directly interacting with the free N termini of the substrates. The extracellular DAP domain of nicastrin, in which the conserved Glu333 residue and the DYIGS motif are critical, is involved in substrate recognition.

Together, the data indicates that the free N terminus of type I membrane proteins resulting from ectodomain shedding is a primary γ-secretase-recognition site.

Discussion

γ-Secretase catalyzes the intramembrane cleavage of amyloid precursor protein (APP) and Notch after their extracellular domains are shed by site-specific proteolysis. Recent developments reveal a growing number of putative type-I membrane proteins participate in cell signaling through γ-secretase dependent regulated intramembrane proteolysis (RIP) events. γ-Secretase appears to have very broad substrate specificity. The only known prerequisites for γ-secretase substrates are (1) type I membrane proteins and (2) shedding the bulk of the extracellular domains from the full-length precursor proteins. Thus, one critical unanswered question concerning γ-secretase mediated RIP is how the γ-secretase complex recognizes its many heterogeneous substrates which seem to have no specific amino acid sequences or substrate recognition domains common amongst them.

One factor important in initial substrate recognition may be the orientation as well as origination of short extracellular domain after the primary processing event. We hypothesize that nicastrin, an essential component of the γ-secretase complex with unknown function may mediate this recognition. Using both *in vivo* and reconstitution assays, we show that the ectodomain of nicastrin physically and functionally binds the new amino terminus that is generated upon proteolysis of the extracellular APP and Notch domains, providing an interaction for recruitment of the substrates into the γ-secretase complex. This interaction requires the ectodomain and not the transmembrane of nicastrin. Deletion of the DAP domain in nicastrin abolished binding of nicastrin to its substrates. Mutation of the conserved Glu333 to Ala affects substrate binding and inhibits

intramembrane proteolysis in vivo. Novel assays developed towards chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduce the binding and cleavage of substrate by γ-secretase. Our results indicate that nicastrin is a receptor for the amino-terminal stubs that are generated by ectodomain shedding of type-I transmembrane proteins. Nicastrin presents these substrates to γ-secretase and thereby facilitates their disposal via intramembrane proteolysis.

Importantly, the work addresses the function and working mechanism of the γ-secretase complex. It establishes the role of nicastrin as a sensor for amino-terminal fragments of the substrates. It provides for the first time an explanation for why transmembrane proteins only become substrates for the complex after their ectodomain is shed. Furthermore, the work introduces novel and innovative approaches that may be used to further analyze and understand the biochemistry of the γ-secretase complex. We propose that γ-secretase evolved in a unique and elegant convergence whereby the substrate recognition function of an aminopeptidase (nicastrin domain) was united with the catalytic function of an intramembrane aspartyl protease (presenilin active site). This enables the γ-secretase complex to execute the unusual events associated with RIP of type-I membrane proteins in the lipid bilayers.

The studies from this chapter reveal that nicastrin has evolved means to recognize short peptide-like extracellular domains of type I membrane proteins with broad specificity. Whether, in some manner, binding properties of nicastrin or the γ-secretase

complex may be differentially regulated remains to be elucidated. Initial evidence supports the fact that binding properties of nicastrin to different substrates may be influenced differentially influenced by number and composition of amino acids of that are present in the extracellular portion of substrates. Similarly, a recent study has demonstrated that certain compounds, including protein kinase inhibitors and their derivatives, act directly on purified γ-secretase to selectively block cleavage of APP but not Notch based substrates (Fraering, Ye et al. 2005). They hypothesize a nucleotide binding site with γ-secretase that can influence the generation of Aβ specifically. Designing and screening for drugs that target γ-secretase mechanisms distinct from the active site may prove to be a more tractable strategy as a therapy for Alzheimer's disease.

Chapter 5

Discussion

A central effort behind this thesis has been an understanding of the molecular mechanisms behind γ-secretase signaling that plays central roles in development and disease. Over the course of this thesis the true identity of all the protein partners involved in this unusual activity were identified. In chapter 2, we demonstrate that in addition to presenilin and nicastrin; APH-1 and PEN-2 are critical components of the large molecular weight complex that performs the aspartyl protease activity of γ-secretase cleavage. In chapter 3, we further characterize APH-1 and provide evidence that conserved GXXXG motifs in its transmembrane plays a critical role in the activity as well as assembly of the γ-secretase complex. In chapter 4, we show that while the transmembrane region of nicastrin is important for complex assembly and activity; the ectodomain residue binds the immediate APP and Notch substrates, providing an interaction for recruitment of the substrates into the γ-secretase complex. In addition, we map specificity of nicastrin interaction with its substrates to a specific conserved glutamate residue to the new amino terminus of substrates, explaining how a repertoire of cell surface receptors undergo remarkably similar processing and analogous nuclear signaling. Taken together, these new data have implications for several areas of biology.

γ-Secretase Complex Identity. Initial exposure to the Rip field revealed limited knowledge regarding the true identity of the components that mediate γ-secretase cleavage. Prior progress in characterizing presenilin and nicastrin aided in the search for additional cofactors. Several criteria were established for candidacy as a member of the γ-secretase complex. First, presenilin orthologues are present from worms to humans, and functional homologues may exist even in plants and unicellular organisms (unpublished

observations). Therefore, obligate protein components of γ-secretase will likely exist in many organisms and at a minimum, they should be present in the genomes from nematodes to humans. Second, the establishment and characterization of the in vitro γ-secretase assay from more defined reconstitution systems as described in this thesis indicates that in at least multicellular organisms, all components in the active enzyme complex are membrane bound. Because γ-secretase activity is unaffected by extensive sodium carbonate washing (which removes non-integral, associated cytoplasmic proteins), all components are likely to contain at least one TM domain. Third, the careful analysis of glycerol velocity gradients, native gels, and gel filtration approaches indicates that the γ-secretase is a ~250 to 900 kDa complex (Yu, Chen et al. 1998; Hebert, Serneels et al. 2004; Evin, Canterford et al. 2005). Presenilin and nicastrin, which are obligatory members, account for ~ 200 kDa, which implies that additional candidate(s) may exist.

In this regard, exhaustive genetic screen in *C.elegans* that identified APH-1 and PEN-2 as candidates seemed intriguing (Francis, McGrath et al. 2002; Goutte, Tsunozaki et al. 2002). Loss-of-function mutations in these genes cause an egg-laying defect identical to loss-of-function mutations in LIN-12 (the *C. elegans* Notch orthologue), APH-2 (the nicastrin orthologue) or SEL-12 (the PS orthologue)(Levitan and Greenwald 1995). The egg laying defect is highly consistent for the disruption of Notch signaling, implicating Aph-1 and PEN-2 in some aspect of the pathway. Additionally, it was reported that the absence of Aph-1 alters Aph2 (nicastrin) distribution in a manner identical to the absence of Sel-12 (Goutte, Tsunozaki et al. 2002). Remarkably Aph-1 and PEN-2 deletions exhibited a similar phenotype, and this strongly suggested that these

genes interacted with Sel-12 and γ-secretase function. As further evidence, APH-1 and PEN-2 are conserved from nematode to humans and is predicted to contain 7 TM and 2 TM domains respectively. They have a predicted molecular weights of ~30 kDa and ~10kDa, thus putatively fulfilling all the criteria for cofactors of the γ-secretase complex enumerated above. Defined biochemical experiments of co-immunoprecipitations, reconstitutions and purification of the complex described in this thesis directly assessed whether these candidates were *bona fide* members of this proteolytic complex. Given the imprecision of the predicted size to the γ-secretase complex: the stoichiometry of each component, as well as other more transient interactors that may modulate this enzyme, remains an area of research interest.

Signal Transduction A Novel Mechanism. Classically, cell-surface receptors transduce extracellular signals via the activation of G-proteins, the gating of ion channels, or alterations in the activity of kinases and phospatases. The recognition that γ-secretase mediated cleavage is a critical step in the signaling mechanism of its substrates has defined a new type of enzyme linked signal transduction. Similarities in Notch and APP processing outlined an emerging model for the normal biological function of γ-secretase. In this generalized model, a receptor protein is first cleaved just outside of the membrane by a disintergrin metalloprotease (ADAM). The scission releases the ectodomain, allowing the membrane-retained, C-terminal fragment to be cleaved by γ-secretase. Release of the intracellular domain (ICD) then permits its translocation to the nucleus, its interaction with appropriate cellular factors and alteration of target gene transcription (Fig 5.1).

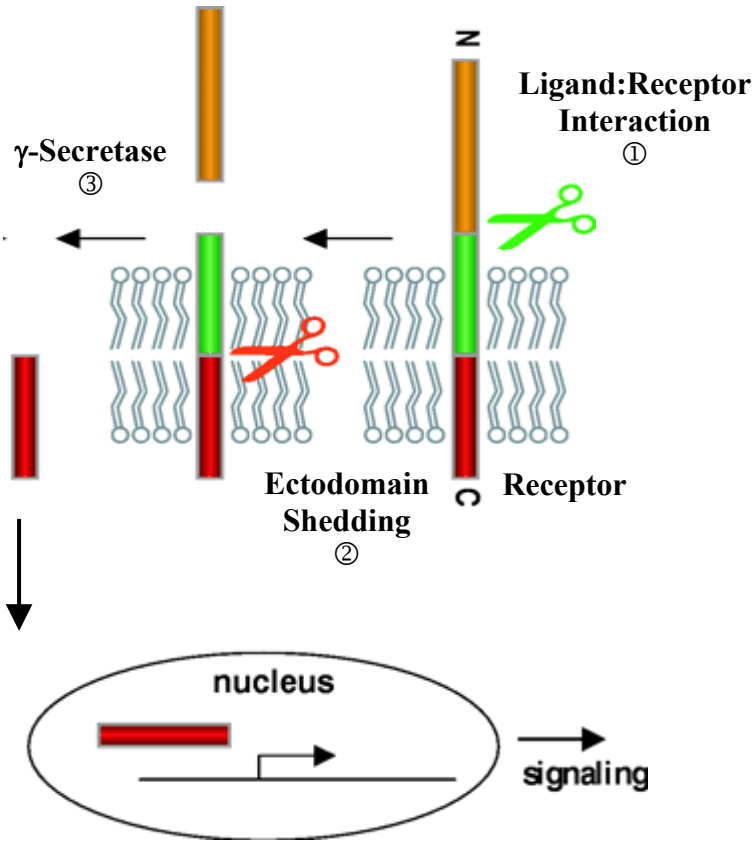


Figure 5.1. A generalized model for γ-secretase-mediated signal transduction. RIP is a signaling process fundamentally controlled by proteases. In the case of gamma secretase mediated RIP, there is a prerequisite shedding of the extracellular domain of group I transmembrane proteins. The remaining membrane tethered fragment becomes a good substrate for gamma-secretase which cleaves within the transmembrane releasing the cytoplasmic tail. The cytoplasmic tail may contain a transcriptional active domain or associated with transcriptional factors that upon cleavage reach the nucleus and thus maybe in a more direct manner modulate gene transcription. Proteases require water to catalyze the hydrolysis of a peptide bond. So in the RIP field, there are unresolved questions as to how these proteases imbeded in an hyddrophobic environment acquire this water. Second, TM are typically alpha helical, a confirmation that makes the backbone amide bond inaccessible to nucleophilic attack because of steric hindrance by amino acid chains.

A cell surface receptor (such as Notch or APP) may bind to its ligand, which permits a disintegrin metalloprotease (ADAM) or other sheddases to proteolytically clip the ectodomain near the plasma membrane. The resultant C-terminal stub then serves as a substrate for γ-secretase, which cuts somewhat heterogeneously within the transmembrane domain. The released intracellular domain (ICD) is then able to translocate to the nucleus and alter gene transcription. For Notch and APP, the respective ICDs interact with a factor that promotes stability.

In addition to Notch and APP, several previously known receptors were discovered to be γ-secretase substrates. Recent developments reveal at least 30 putative type-I membrane proteins participate in cell signaling through γ-secretase dependent regulated intramembrane proteolysis (RIP) events. For example, epithelial cadherin (E-cadherin), a type 1 transmembrane protein that mediates Ca^{2+} dependent cell-cell adhesion and recognition, is cleaved by γ-secretase (Marambaud, Shioi et al. 2002). ErbB-4, an epidermal growth factor receptor that controls cell proliferation and differentiation, also liberates its cytoplasmic domain in a γ-secretase dependent manner (Ni, Murphy et al. 2001). Interestingly, this receptor has a tyrosine kinase domain in its cytoplasmic tail, suggesting that it may in a combinatorial fashion perform two methods of signal transduction: the γ-secretase mediated release of the cytoplasmic tail and tyrosine kinase-mediated phosphorylation of nuclear proteins. One of the ErbB-4 ligands, neuregulin, may also signal via this mechanism(Coolen, van Loo et al. 2005). As in the case of Notch and its ligands Delta/Jagged it may be increasingly likely that group type-I membrane ligand-receptor pairs may utilize γ-secretase to liberate their respective ICDs.

Hydrolysis within Lipid Bilayer. The recognition that γ-secretase-mediated cleavage of APP and Notch takes place within the membrane provided one example of the phenomenon of intramembrane proteolysis. It has remained surprising that the hydrolysis of a peptide bond (requiring a molecule of water) can occur within the hydrophobic environment of the lipid bilayer. In the case of γ-secretase charged aspartate residues in presenilins, position within this hydrophobic environment are able to catalyze

such a reaction. Several other examples of these intramembrane proteases have been appreciated. For example, the cloning of the site 2 protease (S2P) (Rawson, Zelenski et al. 1997) identified the enzyme responsible for the intramembranous proteolysis of the sterol regulatory element binding protein (SREBP). The intramembranous proteolytic activation of transforming growth factor α (TGFα) was attributed to Rhomboid-1, a serine protease whose active site also resides within the membrane (Urban, Lee et al. 2001). Remarkably, the latter protease is conserved throughout evolution from archaea to metazoans, suggesting that this type of mechanism is very widespread. Together, these examples define an emerging concept of regulated intramembrane proteolysis (RIP) (Brown, Ye et al. 2000) that controls the release of membrane-bound fragment of receptors and growth factors.

The discovery of proteases whose active sites apparently reside within the lipid bilayer emphasizes a new aspect of lipids and their effect on protein and enzyme function. The effects of phospholipids in the in vitro γ-secretase assay demonstrate the importance of the lipid environment for the proteolytic function of γ-secretase. Indeed, complex interactions of lipids with the individual polypeptides of the γ-secretase complex may alter its conformation and affect its proteolytic activity. Thus, different membrane characteristics, such as bilayer thickness and fluidity (cholesterol content) represent potential ways in which γ-secretase activity may be modulated. In this regard, it is interesting to note that depletion of cholesterol can reduce Aβ production in primary hippocampal neurons. Alternatively, abnormalities in genes involved in cholesterol

metabolism have been linked to increase the risk of Alzheimer's disease possibly by increasing production of this neurotoxic peptide(Lukiw, Pappolla et al. 2005).

γ-Secretase Evolution. The presence of PS in many model organisms provides the opportunity for speculation on the evolutionary origin of γ-secretase. Its presence in a broad range of species implies the importance of this protein in signal transduction cascades in general and in developmental pathways in particular. A phylogenetic comparison of PS-containing organisms provides a rough approximation of when PS, and perhaps γ-secretase, arose in evolution. Alignment of human PS against existing genomes identifies presenilin homologues in many metazoan species, including chordates (*H. sapiens, M. musculus, D. rerio, X. lavis*) mollusks (*H. locurum*), arthropods (*D. melanogaster*), and nematodes (*C. elegans*). Moreover, the ability of human presenilin to rescue *C. elegans* lacking sel-12 (Levitan, Doyle et al. 1996) indicates the functional conservation of these protein throughout the animal kingdom. The presence of presenilin homologues in plants (*A. thaliana* and *O. Sativa*) suggests that the strategy of intramembrane cleaving proteases probably arose much earlier in eukaryotic evolution. Indeed, the class of intramembrane serine proteases defined by Rhomboid-1 appears to have greater evolutionary conservation. This protease is present in archaea through metazoans, suggesting that intramembrane proteolysis is an ancient strategy that predates the divergence of eukaryotes and archaea (Fig 5.2).

A central process in the formation of multicellular organisms is cell-to-cell signaling. The notch signaling pathway regulates developmental, cell growth, and

differentiation in a wide range of organs from insects to humans. What function could gamma secretase be mediating in unicellular organisms? We hypothesize muticellular

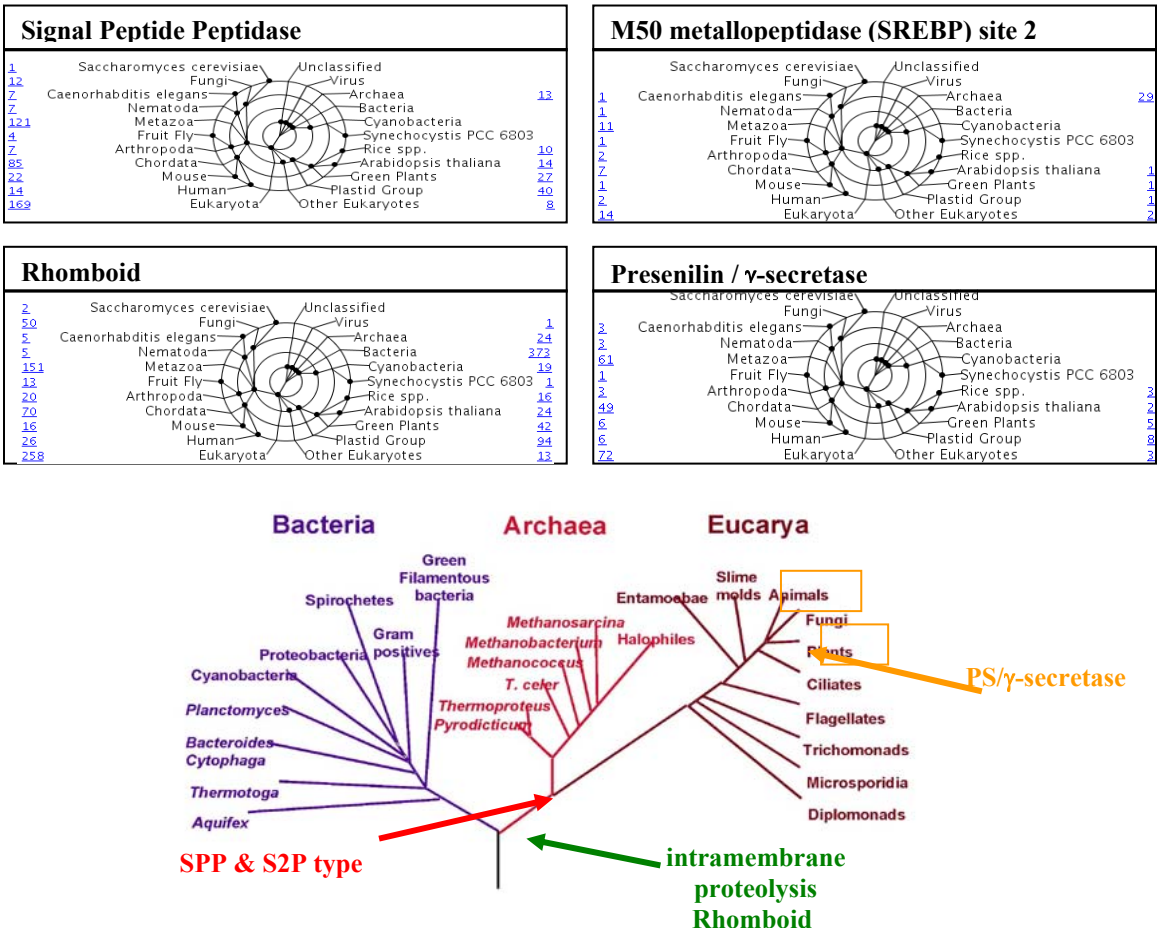


Figure 5.2. Evolutionary conservation of presenilin in plants and animals. Homology of γ-secretase complex components have been previously determined by Blastp search against the currently available genome databases deposited in Genbank. In the case of presenilin, all homologs contain the tow transmembrane aspartates critical to γ-secretase activity. Taxonomic coverage was obtained from the European Bioinformatics Inistitute. A cladogram of life, shows the estimated evolutionary relationship among all known organisms. Presenilin is conserved from animals to plants (but not fungi), consistent with its role in cell fate decisions that complex multicellular organisms require for development. Interestingly, the strategy of intramembranous proteolysis is even more ancient and conserved between the Archaea, Eukaryota, and Bacteria domains, as demonstrated by the discovery of intramembrane-cleaving serine protease Rhomboid-1. Cladogram is adapted from Wikipedia. Referenced web sites http://www.ebi.ac.uk/ & http://en.wikipedia.org/wiki/Phylogenetic_tree.

Figure . Continued

SPP: and potential eukaryotic homologs represent a family of aspartic proteases that promote intramembrane proteolysis to release biologically important peptides. Signal peptide peptidase (SPP) catalyses intramembrane proteolysis of some signal peptides after they have been cleaved from a preprotein. In humans, SPP activity is required to generate signal sequence-derived human lymphocyte antigen-E epitopes that are recognized by the immune system, and are required in the processing of the hepatitis C virus core protein.

Rhomboid: This family contains integral membrane proteins that are related to Drosophila rhomboid protein. Members of this family are found in archaea, bacteria and eukaryotes. Analysis suggests that Rhomboid-1 is a novel intramembrane serine protease that directly cleaves the membrane-anchored TGF-alpha-like growth factor Spitz, allowing it to activate the Drosophila EGF receptor. These proteins contain three strongly conserved histidines in the putative transmembrane regions that may be involved in the peptidase function. This group of proteins contain probable serine peptidases belonging to the MEROPS peptidase family S54 (Rhomboid, clan S-) and proteins classified as non-peptidase homologues that either have been found experimentally to be without peptidase activity, or lack amino acid residues that are believed to be essential for the catalytic activity.

S2P: Members of the M50 metallopeptidase family include mammalian sterol-regulatory element binding protein (SREBP) site 2 proteases and various hypothetical bacterial homologues. SREBPs are membrane-bound transcription factors that regulate cholesterol and fatty acid synthesis in mammalian cells. In order to be activated, the N-terminal domain must be released from the membrane into the nucleus by proteolytic cleavage at two sites: the first site is located within the lumen of the endoplasmic reticulum, and second site within the transmembrane (TM) region. SREBP site 2 proteases act at this second, TM, site. In humans, the protease is encoded by the S2P gene. Once the SREBP has been released into the nucleus, it acts to stimulate transcription of genes involved in lipid metabolism. The site 2 protease has been shown to act on SREBP at the Leu522-Ser523 bond.

Presenilins: are polytopic transmembrane (TM) proteins, mutations in which are associated with the occurrence of early-onset familial Alzheimer's disease, a rare form of the disease that results from a single-gene mutation. They contain and are critical in forming the active site of γ-secretase. Physiological functions of presenilins may be related to developmental signalling, apoptotic signal transduction, or processing of selected proteins, such as the beta-amyloid precursor protein(beta-APP). There are a number of subtypes which belong to this presenilin family. That presenilin homologues have been identified in species that do not have an Alzheimer’s disease correlate suggests that they may have functions unrelated to the disease, homologues having been identified in mouse, Drosophila melanogaster , Caenorhabditis elegans and other members of the eukarya including plants.

development processes may have originated from components and mechanisms that unicellular organisms use for communication amongst each other. These components evolved to operate at the interface between individual cells, i.e. the cell outer membrane, and thus formed the prototypical developmental system used in metazoans. Subsequent improvement and elaboration of this prototype intramembrane protease produced the complex developmental systems critical for multicellular organisms. Ongoing studies in our lab, attempt to elucidate the functional role of conserved γ-secretase components in a unicellular green alga, *Chlamydomonas reinhardtii*, as well as the contributions they may make to the independent evolution of multicellularity in plants, such as *Arabidopsis thaliana*.

Broad Substrate Specificity, the Role of Nicastrin. γ-Secretase catalyzes the intramembrane cleavage of amyloid precursor protein (APP) and Notch after their extracellular domains are shed by site-specific proteolysis. Recent developments reveal a growing number of putative type-I membrane proteins participate in cell signaling through γ-secretase dependent regulated intramembrane proteolysis (RIP) events (Brown, Ye et al. 2000). γ-Secretase appears to have very broad substrate specificity. The only known prerequisites for γ-secretase substrates are (1) type I membrane proteins and (2) shedding the bulk of the extracellular domains from the full-length precursor proteins. Thus, one critical unanswered question concerning γ-secretase mediated RIP remained how the γ-secretase complex recognizes its many heterogeneous substrates which seem to have no specific amino acid sequences or substrate recognition domains common amongst them.

One factor important in initial substrate recognition may be the orientation as well as origination of short extracellular domain after the primary processing event. We hypothesized that nicastrin, an essential component of the γ-secretase complex with unknown function may mediate this recognition. Using both *in vivo* and reconstitution assays, we showed that the ectodomain of nicastrin binds the new amino terminus that is generated upon proteolysis of the extracellular APP and Notch domains, providing an interaction for recruitment of the substrates into the γ-secretase complex. Novel assays developed towards chemical- or antibody-mediated blocking of the free amino terminus, addition of purified nicastrin ectodomain, or mutations in the ectodomain markedly reduced the binding and cleavage of substrate by γ-secretase. Our results indicated that nicastrin is a receptor for the amino-terminal stubs that are generated by ectodomain shedding of type-I transmembrane proteins. Nicastrin presents these substrates to γ-secretase and thereby facilitates their disposal via intramembrane proteolysis (Shah, Lee et al. 2005).

Importantly, the thesis work addressed the function and working mechanism of the γ-secretase complex. It establishes the role of nicastrin as a sensor for amino-terminal fragments of the substrates. It provides for the first time an explanation for why transmembrane proteins only become substrates for the complex after their ectodomain is shed. Furthermore, the work introduces novel and innovative approaches that may be used to further analyze and understand the biochemistry of the γ-secretase complex. We propose that γ-secretase evolved in a unique and elegant convergence whereby the substrate recognition function of an aminopeptidase (nicastrin domain) was united with

the catalytic function of an intramembrane aspartyl protease (presenilin active site). This enables the γ-secretase complex to execute the unusual events associated with RIP of type-I membrane proteins in the lipid bilayers.

APP Function. Although intensively studied now for numerous years, this protein remains without a defined function. The fact that all known substrates of gamma-secretase to date are single-TM cell surface receptors has implications for APP. The overexpression of the APP intracellular domain (AICD) resulted in its nuclear translocation, created the possibility that APP possesses a similar receptor function as other γ-secretase substrates (Cao and Sudhof 2001). What function may AICD be mediating in these cells? Comparative analyses among several model organisms provide circumstantial evidence that APP (and perhaps AICD in particular) plays a role in the genesis and/or remodeling of synapses. It is chiefly expressed in neurons, although some isoforms can be found ubiquitously expressed in mammals. In Drosophila, the APP-like protein (APPL) is exclusively expressed in post-mitototic neurons and in humans and rats; there is a brain specific isoform (APP695).

More detailed spatial and temporal analyses support an axonal and synaptic distribution of APP. First, APP reaches maximal expression in neurons at the period of greatest synaptic formation (2 weeks postnatally), and it undergoes fast anterograde transport to axon terminal and synaptic sites. Second, APP has been directly visualized in a subset of synapses within human and rat brain and the neuromuscular junction(Torroja, Chu et al. 1999). Third, in the fly, APPL is enriched in specific layers of the optic lobes

where synapses are actively forming. As would be expected for AICD-mediated signaling, substantial proteolytic processing of APP is also found at these sites. Whereas full-length APP is predominantly associated with periods of axonogenesis, increases in APPs secretion appear to occur at the time of end-arbor formation in rat brain (Moya, Benowitz et al. 1994). One possible interpretation of these findings is that increased ligand-dependent ADAM cleavage occurs at synapses, a predicted requirement for this type of signal transduction.

Transgenic and knockout analyses of APP provide more direct support for its role in synaptic function. Ablation of APP in mice results in cerebral gliosis and subtle locomotor defects. Similarly, APPL- flies display only a mild behavioral deficit that was rescued by overexpression of human APP (Luo, Tully et al. 1992). Nevertheless, more detailed characterization of both animal models has revealed alteration in synaptic integrity. APP-/- mice exhibit reduced immunoreactivity for the synaptic markers synapsin and synaptophysin, as well as impairments in long-term potentiation (Dawson, Seabrook et al. 1999). Flies lacking APPL exhibit a small but significant loss in synaptic bouton number in the neuromuscular junction (NMJ) (Torroja, Chu et al. 1999). Conversely, the transgenic overexpression of APP in either animal result in increased numbers of synapses. In mice transgenic for wild-type APP, significant increase in the number of synaptophsin immunoreactive presynaptic terminals is observed. Similarly, when APPL is overexpressed in fly motor neurons, a marked increase in synapses is observed at the neuromuscular junction (Torroja, Chu et al. 1999). Whether this represents an increase in synapse formation or a decrease in synaptic pruning remains to

be determined. However, the combined data argue strongly that APP can modulate aspects of synaptic formation. Continued experiments that directly test the in vivo function of AICD (and cognate binding partners) will help towards defining a function.

γ-Secretase, Alzheimer's Disease Treatment and Prevention. Ironically and unfortunately, γ-secretase is critical for developmental decisions at the beginning of life, it is also central to disease pathogenesis at the end of life. The excessive accumulation of Aβ is a central hallmark and causative agent in AD, and this process is mediated by γ-secretase. Thus, partial inhibition of this protease remains one potential strategy for the prevention of AD. However, the recent recognition that γ-secretase cleaves a growing list of group I type transmembrane proteins raises the concern that therapeutic inhibition of this protease may cause unacceptable side effects. γ-Secretase inhibitors therefore require meticulous preclinical and clinical evaluation. The role of γ-secretase in several signal transduction pathways does not necessarily preclude its utility as an AD therapy. It remains possible that a partial inhibition of γ-secretase may reduce Aβ production sufficiently to prevent the progression of disease in the final 10 to 30 years of life. In this regard, chronic partial γ-secretase inhibition would be conceptually similar to cholesterol-lowering agents that are widely used to prevent heart disease. For example, partial (30 to 50%) inhibition with the "statin" class of inhibitors reduces blood cholesterol, has few side effects and significantly lowers the risk of cardiovascular disease. Candidate γ-secretase inhibitors at certain doses might act in a similar way, lowering Aβ just enough to reduce the risk of developing AD while maintaining enough activity to be compatible for other biological functions.

Alzheimer's disease remains an enormous, unmet medical need. As AD prevalence increases exponentially with age, drugs that provide a modest 5-year delay in the onset of symptoms are calculated to reduce the number of affected people by as much as 50%. Considerable advance in early diagnosis and new biomarkers coupled with increase knowledge of the culprits involved in this debilitating disease has increased optimism on the therapeutic front. For example, γ-Secretase inhibitors may be useful in conjunction with other emerging AD therapies. Drugs may be useful in combination therapy with β-secretase inhibitors. Because β-secretase activity proceed γ-secretase cleavage in the generation of Aβ, the combined inhibition of both enzymes would likely be synergistic and therefore would require decreased dosages (and thus reduce side effects) for each. Alternatively, strategies to decrease aggregation of Aβ or alter production of fragment length of Aβ using agents such as statins and nonsteroidal anti-inflammatories are being tested. Ultimately and hopefully leading to a cocktail of treatments directed at a range of AD targets.

Over the course of my graduate studentship the identification of the members of γ-secretase complex, aspects of its assembly, and recognition of substrate binding sites distinct from the active site reveal additional protein targets for drug development. As the biology and function of the protease complex is better understood, it is plausible that agents directed outside of the active site that may mediate substrate selectivity for APP proteolysis as compared to other substrates could be targeted. Alzheimer's therapies remain a "must win" disease target for pharma and more importantly an increasing

ageing society. Future studies of the γ-secretase protease, its role as a signaling hub or as a controller of a repertoire of membrane proteins should continue to reveal exciting new insights into its role in normal biology and disease.

References

Annaert, W. and B. De Strooper (1999). "Presenilins: molecular switches between proteolysis and signal transduction." Trends in Neurosciences **22**(10): 439-443.

Annaert, W. G., L. Levesque, et al. (1999). "Presenilin 1 Controls {gamma}-Secretase Processing of Amyloid Precursor Protein in Pre-Golgi Compartments of Hippocampal Neurons." J. Cell Biol. **147**(2): 277-294.

Arriagada, P. V., K. Marzloff, et al. (1992). "Distribution of Alzheimer-Type Pathological-Changes in Nondemented Elderly Individuals Matches the Pattern in Alzheimers-Disease." Neurology **42**(9): 1681-1688.

Artavanis-Tsakonas, S., M. D. Rand, et al. (1999). "Notch Signaling: Cell Fate Control and Signal Integration in Development." Science **284**(5415): 770-776.

Asai, M., C. Hattori, et al. (2003). "Putative function of ADAM9, ADAM10, and ADAM17 as APP [alpha]-secretase." Biochemical and Biophysical Research Communications **301**(1): 231-235.

Berrios, G. E. (1990). "Alzheimers-Disease - a Conceptual History." International Journal of Geriatric Psychiatry **5**(6): 355-365.

Brown, M. S., J. Ye, et al. (2000). "Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans." Cell **100**(4): 391-8.

Burdick, D., B. Soreghan, et al. (1992). "Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs." J. Biol. Chem. **267**(1): 546-554.

Buxbaum, J. D., K. N. Liu, et al. (1998). "Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor." Journal of Biological Chemistry **273**(43): 27765-27767.

Buxbaum, J. D., G. Thinakaran, et al. (1998). "Alzheimer Amyloid Protein Precursor in the Rat Hippocampus: Transport and Processing through the Perforant Path." J. Neurosci. **18**(23): 9629-9637.

Cai, H., Y. Wang, et al. (2001). "BACE1 is the major beta-secretase for generation of Abeta peptides by neurons." Nat Neurosci **4**(3): 233-4.

Cai, X. D., T. E. Golde, et al. (1993). "Release of Excess Amyloid Beta-Protein from a Mutant Amyloid Beta-Protein Precursor." Science **259**(5094): 514-516.

Campion, D., C. Dumanchin, et al. (1999). "Early-onset autosomal dominant Alzheimer disease: Prevalence, genetic heterogeneity, and mutation spectrum." American Journal of Human Genetics **65**(3): 664-670.

Cao, X. and T. C. Sudhof (2001). "A Transcriptively Active Complex of APP with Fe65 and Histone Acetyltransferase Tip60." Science **293**(5527): 115-120.

Capell, A., J. Grunberg, et al. (1998). "The Proteolytic Fragments of the Alzheimer's Disease-associated Presenilin-1 Form Heterodimers and Occur as a 100-150-kDa Molecular Mass Complex." J. Biol. Chem. **273**(6): 3205-3211.

Chen, F., G. Yu, et al. (2001). "Nicastrin binds to membrane-tethered Notch." Nat Cell Biol **3**(8): 751-4.

Citron, M., T. Oltersdorf, et al. (1992). "Mutation of the Beta-Amyloid Precursor Protein in Familial Alzheimers-Disease Increases Beta-Protein Production." Nature **360**(6405): 672-674.

Citron, M., D. B. Teplow, et al. (1995). "Generation of Amyloid-Beta Protein from Its Precursor Is Sequence-Specific." Neuron **14**(3): 661-670.

Cook, D. G., M. S. Forman, et al. (1997). "Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells." Nature Medicine **3**(9): 1021-1023.

Coolen, M. W., K. M. J. van Loo, et al. (2005). "Reduced Aph-1b expression causes tissue- and substrate-specific changes in γ-secretase activity in rats with a complex phenotype." FASEB J.: 05-4337fje.

Cruts, M., C. M. van Duijn, et al. (1998). "Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease." Hum. Mol. Genet. **7**(1): 43-51.

Dann, C. E., J.-C. Hsieh, et al. (2001). "Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains." Nature **412**(6842): 86-90.

Dawson, G. R., G. R. Seabrook, et al. (1999). "Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the [beta]-amyloid precursor protein." Neuroscience **90**(1): 1-13.

De Jonghe, C., C. Esselens, et al. (2001). "Pathogenic APP mutations near the {gamma}-secretase cleavage site differentially affect A{beta} secretion and APP C-terminal fragment stability." Hum. Mol. Genet. **10**(16): 1665-1671.

De Strooper, B., W. Annaert, et al. (1999). "A presenilin-1-dependent [gamma]-secretase-like protease mediates release of Notch intracellular domain." Nature **398**(6727): 518-522.

De Strooper, B., P. Saftig, et al. (1998). "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein." Nature **391**(6665): 387-390.

Dickson, D. W., H. A. Crystal, et al. (1995). "Correlations of synaptic and pathological markers with cognition of the elderly." Neurobiology of Aging **16**(3): 285-298.

Domeniconi, M., N. Zampieri, et al. (2005). "MAG Induces Regulated Intramembrane Proteolysis of the p75 Neurotrophin Receptor to Inhibit Neurite Outgrowth." Neuron **46**(6): 849-855.

Donoviel, D. B., A.-K. Hadjantonakis, et al. (1999). "Mice lacking both presenilin genes exhibitearlyembryonic patterningdefects." Genes Dev. **13**(21): 2801-2810.

Efthimiopoulos, S., E. Floor, et al. (1998). "Enrichment of Presenilin 1 Peptides in Neuronal Large Dense-Core and Somatodendritic Clathrin-Coated Vesicles." Journal of Neurochemistry **71**(6): 2365-2372.

Esch, F. S., P. S. Keim, et al. (1990). "Cleavage of Amyloid-Beta Peptide During Constitutive Processing of Its Precursor." Science **248**(4959): 1122-1124.

Esler, W. P., W. T. Kimberly, et al. (2002). "Activity-dependent isolation of the presenilin- gamma -secretase complex reveals nicastrin and a gamma substrate." Proc Natl Acad Sci U S A **99**(5): 2720-5.

Evin, G., L. D. Canterford, et al. (2005). "Transition-state analogue gamma-secretase inhibitors stabilize a 900 kDa presenilin/nicastrin complex." Biochemistry **44**(11): 4332-41.

Farmery, M. R., L. O. Tjernberg, et al. (2003). "Partial Purification and Characterization of [Gamma]-Secretase from Post-mortem Human Brain." Journal of Biological Chemistry **278**(27): 24277-24284.

Farzan, M., C. E. Schnitzler, et al. (2000). "BACE2, a beta -secretase homolog, cleaves at the beta site and within the amyloid-beta region of the amyloid-beta precursor protein." PNAS **97**(17): 9712-9717.

Fleming, K. G. and D. M. Engelman (2001). "Specificity in transmembrane helix-helix interactions can define a hierarchy of stability for sequence variants." PNAS **98**(25): 14340-14344.

Fraering, P. C., W. Ye, et al. (2005). "{gamma}-Secretase Substrate Selectivity Can Be Modulated Directly via Interaction with a Nucleotide-binding Site." J. Biol. Chem. **280**(51): 41987-41996.

{gamma} -Secretase is an unusual protease with an intramembrane catalytic site that cleaves many type I membrane proteins, including the amyloid {beta}-protein (A{beta}) precursor (APP) and the Notch receptor. Genetic and biochemical studies have identified four membrane proteins as components of {gamma}-secretase: heterodimeric presenilin composed of its N- and C-terminal fragments, nicastrin, Aph-1, and Pen-2. Here we demonstrated that certain compounds, including protein kinase inhibitors and their derivatives, act directly on purified {gamma}-secretase to selectively block cleavage of APP- but not Notch-based substrates. Moreover, ATP activated the generation of the APP intracellular domain and A{beta}, but not the generation of the Notch intracellular domain by the purified protease complex, and was a direct competitor of the APP-selective inhibitors, as were other nucleotides. In accord, purified {gamma}-secretase bound specifically to an ATP-linked resin. Finally, a photoactivable ATP analog specifically labeled presenilin 1-C-terminal fragments in purified {gamma}-secretase preparations; the labeling was blocked by ATP itself and APP-selective {gamma}-secretase inhibitors. We concluded that a nucleotide-binding site exists within {gamma}-secretase, and certain compounds that bind to this site can specifically modulate the generation of A{beta} while sparing Notch. Drugs targeting the {gamma}-secretase nucleotide-binding site represent an attractive strategy for safely treating Alzheimer disease.

Francis, R., G. McGrath, et al. (2002). "aph-1 and pen-2 Are Required for Notch Pathway Signaling, [gamma]-Secretase Cleavage of [beta]APP, and Presenilin Protein Accumulation." Developmental Cell **3**(1): 85-97.

Galarneau, A., M. Primeau, et al. (2002). "[beta]-Lactamase protein fragment complementation assays as in vivo and in vitro sensors of protein-protein interactions." Nat Biotech **20**(6): 619-622.

Games, D., D. Adams, et al. (1995). "Alzheimer-Type Neuropathology in Transgenic Mice Overexpressing V717f Beta-Amyloid Precursor Protein." Nature **373**(6514): 523-527.

Goutte, C., M. Tsunozaki, et al. (2002). "APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos." PNAS **99**(2): 775-779.

Grunberg, J., J. Walter, et al. (1998). "Alzheimer's Disease Associated Presenilin-1 Holoprotein and Its 18-20 kDa C-Terminal Fragment Are Death Substrates for Proteases of the Caspase Family." Biochemistry **37**(8): 2263-2270.

Gu, Y., F. Chen, et al. (2003). "APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes." J Biol Chem **278**(9): 7374-80.

Haass, C. (1997). "Presenilins: Genes for life and death." Neuron **18**(5): 687-690.

Hardy, J. (1997). "Amyloid, the presenilins and Alzheimer's disease." Trends in Neurosciences **20**(4): 154-159.

Harper, J. D., C. M. Lieber, et al. (1997). "Atomic force microscopic imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-beta protein." Chemistry & Biology **4**(12): 951-959.

Harper, J. D., S. S. Wong, et al. (1997). "Observation of metastable A beta amyloid protofibrils by atomic force microscopy." Chemistry & Biology **4**(2): 119-125.

Hartley, D. M., D. M. Walsh, et al. (1999). "Protofibrillar Intermediates of Amyloid beta -Protein Induce Acute Electrophysiological Changes and Progressive Neurotoxicity in Cortical Neurons." J. Neurosci. **19**(20): 8876-8884.

Hartmann, T., S. C. Bieger, et al. (1997). "Distinct sites of intracellular production for Alzheimer's disease A beta 40/42 amyloid peptides." Nature Medicine **3**(9): 1016-1020.

Hebert, S. S., L. Serneels, et al. (2004). "Coordinated and widespread expression of [gamma]-secretase in vivo: evidence for size and molecular heterogeneity." Neurobiology of Disease **17**(2): 260-272.

Herreman, A., D. Hartmann, et al. (1999). "Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency." PNAS **96**(21): 11872-11877.

Herreman, A., L. Serneels, et al. (2000). "Total inactivation of [ggr]-secretase activity in presenilin-deficient embryonic stem cells." Nat Cell Biol **2**(7): 461-462.

Hsia, A. Y., E. Masliah, et al. (1999). "Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models." PNAS **96**(6): 3228-3233.

Hsiao, K., P. Chapman, et al. (1996). "Correlative memory deficits, A beta elevation, and amyloid plaques in transgenic mice." Science **274**(5284): 99-102.

Hu, Y. and M. E. Fortini (2003). "Different cofactor activities in gamma-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex." J Cell Biol **161**(4): 685-90.

Hu, Y., Y. Ye, et al. (2002). "Nicastrin Is Required for [gamma]-Secretase Cleavage of the Drosophila Notch Receptor." Developmental Cell **2**(1): 69-78.

Hussain, I., D. Powell, et al. (1999). "Identification of a Novel Aspartic Protease (Asp 2) as [beta]-Secretase." Molecular and Cellular Neuroscience **14**(6): 419-427.

Hutton, M. and J. Hardy (1997). "The presenilins and Alzheimer's disease." Hum. Mol. Genet. **6**(10): 1639-1646.

Ikeuchi, T. and S. S. Sisodia (2003). "The Notch Ligands, Delta1 and Jagged2, Are Substrates for Presenilin-dependent "gamma -Secretase" Cleavage." J. Biol. Chem. **278**(10): 7751-7754.

Irizarry, M. C., F. Soriano, et al. (1997). "Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse." J Neurosci **17**(18): 7053-9.

Iwatsubo, T., A. Odaka, et al. (1994). "Visualization of a-Beta-42(43) and a-Beta-40 in Senile Plaques with End-Specific a-Beta Monoclonals - Evidence That an Initially Deposited Species Is a-Beta-42(43)." Neuron **13**(1): 45-53.

Jarrett, J. T., E. P. Berger, et al. (1993). "The Carboxy Terminus of the Beta-Amyloid Protein Is Critical for the Seeding of Amyloid Formation - Implications for the Pathogenesis of Alzheimers-Disease." Biochemistry **32**(18): 4693-4697.

Jarriault, S., C. Brou, et al. (1995). "Signalling downstream of activated mammalian Notch." Nature **377**(6547): 355-358.

Kang, J., H. G. Lemaire, et al. (1987). "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor." Nature **325**(6106): 733-6.

Kim, D. Y., L. A. M. Ingano, et al. (2002). "Nectin-1alpha , an Immunoglobulin-like Receptor Involved in the Formation of Synapses, Is a Substrate for Presenilin/gamma -Secretase-like Cleavage." J. Biol. Chem. **277**(51): 49976-49981.

Kim, S.-H. and S. S. Sisodia (2005). "Evidence That the "NF" Motif in Transmembrane Domain 4 of Presenilin 1 Is Critical for Binding with PEN-2." J. Biol. Chem. **280**(51): 41953-41966.

Kim, T.-W., W. H. Pettingell, et al. (1997). "Endoproteolytic Cleavage and Proteasomal Degradation of Presenilin 2in Transfected Cells." J. Biol. Chem. **272**(17): 11006-11010.

Kim, T.-W., W. H. Pettingell, et al. (1997). "Alternative Cleavage of Alzheimer-Associated Presenilins During Apoptosis by a Caspase-3 Family Protease." Science **277**(5324): 373-376.

Kimberly, W. T., M. J. LaVoie, et al. (2003). "Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2." Proc Natl Acad Sci U S A **100**(11): 6382-7.

Kimberly, W. T., J. B. Zheng, et al. (2001). "The Intracellular Domain of the beta -Amyloid Precursor Protein Is Stabilized by Fe65 and Translocates to the Nucleus in a Notch-like Manner." J. Biol. Chem. **276**(43): 40288-40292.

Kleiger, G. and D. Eisenberg (2002). "GXXXG and GXXXA Motifs Stabilize FAD and NAD(P)-binding Rossmann Folds Through C[alpha]-H...O Hydrogen Bonds and van der Waals Interactions." Journal of Molecular Biology **323**(1): 69-76.

Klyubin, I., D. M. Walsh, et al. (2005). "Amyloid [beta] protein immunotherapy neutralizes A[beta] oligomers that disrupt synaptic plasticity in vivo." Nat Med **11**(5): 556-561.

Knowles, R. B., T. Gomez-Isla, et al. (1998). "A beta associated neuropil changes: Correlation with neuronal loss and dementia." Journal of Neuropathology and Experimental Neurology **57**(12): 1122-1130.

Knowles, R. B., C. Wyart, et al. (1999). "Plaque-induced neurite abnormalities: Implications for disruption of neural networks in Alzheimer's disease." PNAS **96**(9): 5274-5279.

Kornilova, A. Y., F. Bihel, et al. (2005). "The initial substrate-binding site of {gamma}-secretase is located on presenilin near the active site." PNAS **102**(9): 3230-3235.

{gamma} -Secretase is a structurally enigmatic multiprotein complex that catalyzes intramembrane proteolysis of a variety of substrates, including the amyloid {beta}-protein precursor of Alzheimer's disease and the Notch receptor essential to cell differentiation. The active site of this transmembrane aspartyl protease apparently lies at the interface between two subunits of presenilin-1 (PS1); however, evidence suggests the existence of an initial substrate-binding site that is distinct from the active site. Here, we report that photoaffinity probes based on potent helical peptide inhibitors and designed to mimic the amyloid {beta}-protein precursor substrate bind specifically to the PS subunit interface, at a site close to the active site. The location of the helical peptide-binding site suggests that substrate passes between the two PS1 subunits to access the active site. An aggressive Alzheimer-causing mutation in PS1 strongly reduced photolabeling by a transition-state analogue but not by helical peptides, providing biochemical evidence that the pathological effect of this PS mutation is due to alteration of the active-site topography.

Kovacs, D. M., H. J. Fausett, et al. (1996). "Alzheimer-associated presenilins 1 and 2: Neuronal expression in brain and localization to intracellular membranes in mammalian cells." Nature Medicine **2**(2): 224-229.

Lah, J. J., C. J. Heilman, et al. (1997). "Light and Electron Microscopic Localization of Presenilin-1 in Primate Brain." J. Neurosci. **17**(6): 1971-1980.

Lammich, S., M. Okochi, et al. (2002). "Presenilin-dependent Intramembrane Proteolysis of CD44 Leads to the Liberation of Its Intracellular Domain and the Secretion of an Abeta -like Peptide." J. Biol. Chem. **277**(47): 44754-44759.

Leahy, D. J., I. I. I. C. E. Dann, et al. (2000). "A Mammalian Expression Vector for Expression and Purification of Secreted Proteins for Structural Studies." Protein Expression and Purification **20**(3): 500-506.

Lee, S.-F., S. Shah, et al. (2004). "A Conserved GXXXG Motif in APH-1 Is Critical for Assembly and Activity of the {gamma}-Secretase Complex." J. Biol. Chem. **279**(6): 4144-4152.

Lee, S. F., S. Shah, et al. (2002). "Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch." J Biol Chem **277**(47): 45013-9.

Levitan, D., Timothy G. Doyle, et al. (1996). "Assessment of normal and mutant human presenilin function in Caenorhabditis elegans." PNAS **93**(25): 14940-14944.

Levitan, D. and I. Greenwald (1995). "Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene." Nature **377**(6547): 351-354.

Lleo, A., O. Berezovska, et al. (2003). "Notch1 Competes with the Amyloid Precursor Protein for {gamma}-Secretase and Down-regulates Presenilin-1 Gene Expression." J. Biol. Chem. **278**(48): 47370-47375.

Logeat, F., C. Bessia, et al. (1998). "The Notch1 receptor is cleaved constitutively by a furin-like convertase." PNAS **95**(14): 8108-8112.

Lorenzo, A. and B. A. Yankner (1994). "{beta}-Amyloid Neurotoxicity Requires Fibril Formation and is Inhibited by Congo Red." PNAS **91**(25): 12243-12247.

Lukiw, W. J., M. Pappolla, et al. (2005). "Alzheimerâ€™s Diseaseâ€"A Dysfunction in Cholesterol and Lipid Metabolism." Cellular and Molecular Neurobiology **25**(3 - 4): 475-483.

Luo, L., T. Tully, et al. (1992). "Human amyloid precursor protein ameliorates behavioral deficit of flies deleted for appl gene." Neuron **9**(4): 595-605.

Luo, W.-j., H. Wang, et al. (2003). "PEN-2 and APH-1 Coordinately Regulate Proteolytic Processing of Presenilin 1." J. Biol. Chem. **278**(10): 7850-7854.

MacKenzie, K. R., J. H. Prestegard, et al. (1997). "A Transmembrane Helix Dimer: Structure and Implications." Science **276**(5309): 131-133.

Marambaud, P., J. Shioi, et al. (2002). "A presenilin-1/{gamma}-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions." EMBO J. **21**(8): 1948-1956.

Marambaud, P., P. H. Wen, et al. (2003). "A CBP Binding Transcriptional Repressor Produced by the PS1/[epsilon]-Cleavage of N-Cadherin Is Inhibited by PS1 FAD Mutations." Cell **114**(5): 635-645.

Maretzky, T., M. Schulte, et al. (2005). "L1 Is Sequentially Processed by Two Differently Activated Metalloproteases and Presenilin/{gamma}-Secretase and Regulates Neural Cell Adhesion, Cell Migration, and Neurite Outgrowth." Mol. Cell. Biol. **25**(20): 9040-9053.

Mattson, M. P. (1997). "Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives." Physiol. Rev. **77**(4): 1081-1132.

Maurer, K., S. Volk, et al. (1997). "Auguste D and Alzheimer's disease." Lancet **349**(9064): 1546-1549.

May, P., Y. K. Reddy, et al. (2002). "Proteolytic Processing of Low Density Lipoprotein Receptor-related Protein Mediates Regulated Release of Its Intracellular Domain." J. Biol. Chem. **277**(21): 18736-18743.

Morgan, D., D. M. Diamond, et al. (2000). "A[beta] peptide vaccination prevents memory loss in an animal model of Alzheimer's disease." Nature **408**(6815): 982-985.

Moya, K. L., L. I. Benowitz, et al. (1994). "The Amyloid Precursor Protein Is Developmentally Regulated and Correlated with Synaptogenesis." Developmental Biology **161**(2): 597-603.

Mumm, J. S., E. H. Schroeter, et al. (2000). "A Ligand-Induced Extracellular Cleavage Regulates [gamma]-Secretase-like Proteolytic Activation of Notch1." Molecular Cell **5**(2): 197-206.

Ni, C.-Y., M. P. Murphy, et al. (2001). "gamma -Secretase Cleavage and Nuclear Localization of ErbB-4 Receptor Tyrosine Kinase." Science **294**(5549): 2179-2181.

Oddo, S., A. Caccamo, et al. (2003). "Triple-Transgenic Model of Alzheimer's Disease with Plaques and Tangles: Intracellular A[beta] and Synaptic Dysfunction." Neuron **39**(3): 409-421.

Okochi, M., H. Steiner, et al. (2002). "Presenilins mediate a dual intramembranous {gamma}-secretase cleavage of Notch-1." EMBO J. **21**(20): 5408-5416.

Paganetti, P., V. Calanca, et al. (2005). "{beta}-site specific intrabodies to decrease and prevent generation of Alzheimer's A{beta} peptide." J. Cell Biol. **168**(6): 863-868.

Endoproteolysis of the {beta}-amyloid precursor protein (APP) by {beta}- and {gamma}-secretases generates the toxic amyloid {beta}-peptide (A{beta}), which accumulates in the brain of Alzheimer's disease (AD) patients. Here, we established a novel approach to regulate production of A{beta} based on intracellular expression of single chain antibodies (intrabodies) raised to an epitope adjacent to the {beta}-secretase cleavage site of human APP. The intrabodies rapidly associated, within the endoplasmic reticulum (ER), with newly synthesized APP. One intrabody remained associated during APP transport along the secretory line, shielded the {beta}-secretase cleavage site and facilitated the alternative, innocuous cleavage operated by {alpha}-secretase. Another killer intrabody with an ER retention sequence triggered APP disposal from the ER. The first intrabody drastically inhibited and the second almost abolished generation of A{beta}. Intrabodies association with specific substrates rather than with enzymes, may modulate intracellular processes linked to disease with highest specificity and may become instrumental to investigate molecular mechanisms of cellular events.

Parks, A. L., K. M. Klueg, et al. (2000). "Ligand endocytosis drives receptor dissociation and activation in the Notch pathway." Development **127**(7): 1373-1385.

Pike, C. J., A. J. Walencewicz, et al. (1991). "Aggregation-Related Toxicity of Synthetic Beta-Amyloid Protein in Hippocampal Cultures." European Journal of Pharmacology-Molecular Pharmacology Section **207**(4): 367-368.

Popot, J.-L. and D. M. Engelman (2000). "HELICAL MEMBRANE PROTEIN FOLDING, STABILITY, AND EVOLUTION." Annual Review of Biochemistry **69**(1): 881-922.

Rand, M. D., L. M. Grimm, et al. (2000). "Calcium Depletion Dissociates and Activates Heterodimeric Notch Receptors." Mol. Cell. Biol. **20**(5): 1825-1835.

Rawson, R. B., N. G. Zelenski, et al. (1997). "Complementation Cloning of S2P, a Gene Encoding a Putative Metalloprotease Required for Intramembrane Cleavage of SREBPs." Molecular Cell **1**(1): 47-57.

Scheuner, D., C. Eckman, et al. (1996). "Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease." Nature Medicine **2**(8): 864-870.

Schroeter, E. H., J. A. Kisslinger, et al. (1998). "Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain." Nature **393**(6683): 382-386.

Schulz, J. G., W. Annaert, et al. (2003). "Syndecan 3 Intramembrane Proteolysis Is Presenilin/{gamma}-Secretase-dependent and Modulates Cytosolic Signaling." J. Biol. Chem. **278**(49): 48651-48657.

Schwarzman, A. L., N. Singh, et al. (1999). "Endogenous presenilin 1 redistributes to the surface of lamellipodia upon adhesion of Jurkat cells to a collagen matrix." PNAS **96**(14): 7932-7937.

Seubert, P., C. Vigopelfrey, et al. (1992). "Isolation and Quantification of Soluble Alzheimers Beta-Peptide from Biological-Fluids." Nature **359**(6393): 325-327.

Shah, S., S. F. Lee, et al. (2005). "Nicastrin functions as a gamma-secretase-substrate receptor." Cell **122**(3): 435-47.

Shen, J., R. T. Bronson, et al. (1997). "Skeletal and CNS defects in Presenilin-1-deficient mice." Cell **89**(4): 629-639.

Sinha, S., J. P. Anderson, et al. (1999). "Purification and cloning of amyloid precursor protein [beta]-secretase from human brain." Nature **402**(6761): 537-540.

Sisodia, S. S. (1992). "Beta-Amyloid Precursor Protein Cleavage by a Membrane-Bound Protease." Proceedings of the National Academy of Sciences of the United States of America **89**(13): 6075-6079.

Steiner, H., A. Capell, et al. (1998). "Expression of Alzheimer's Disease-associated Presenilin-1 Is Controlled by Proteolytic Degradation and Complex Formation." J. Biol. Chem. **273**(48): 32322-32331.

Strooper, B. D. and W. Annaert (2001). "Presenilins and the intramembrane proteolysis of proteins: facts and fiction." Nat Cell Biol **3**(10): E221-E225.

Struhl, G. and A. Adachi (2000). "Requirements for Presenilin-Dependent Cleavage of Notch and Other Transmembrane Proteins." Molecular Cell **6**(3): 625-636.

Suzuki, N., T. T. Cheung, et al. (1994). "An Increased Percentage of Long Amyloid-Beta Protein Secreted by Familial Amyloid-Beta Protein-Precursor (Beta-App(717)) Mutants." Science **264**(5163): 1336-1340.

Takashima, A., M. Sato, et al. (1996). "Localization of Alzheimer-Associated Presenilin 1 in Transfected COS-7 Cells." Biochemical and Biophysical Research Communications **227**(2): 423-426.

Takasugi, N., T. Tomita, et al. (2003). "The role of presenilin cofactors in the [gamma]-secretase complex." Nature **422**(6930): 438-441.

Taniguchi, Y., S.-H. Kim, et al. (2003). "Presenilin-dependent "{gamma}-Secretase" Processing of Deleted in Colorectal Cancer (DCC)." J. Biol. Chem. **278**(33): 30425-30428.

Terry, R. D. (1996). "The pathogenesis of Alzheimer disease: An alternative to the amyloid hypothesis." Journal of Neuropathology and Experimental Neurology **55**(10): 1023-1025.

Thinakaran, G., D. R. Borchelt, et al. (1996). "Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo." Neuron **17**(1): 181-190.

Thinakaran, G., C. L. Harris, et al. (1997). "Evidence That Levels of Presenilins (PS1 and PS2) Are Coordinately Regulated by Competition for Limiting Cellular Factors." J. Biol. Chem. **272**(45): 28415-28422.

Torroja, L., H. Chu, et al. (1999). "Neuronal overexpression of APPL, the Drosophila homologue of the amyloid precursor protein (APP), disrupts axonal transport." Current Biology **9**(9): 489-493.

Urban, S., J. R. Lee, et al. (2001). "Drosophila Rhomboid-1 Defines a Family of Putative Intramembrane Serine Proteases." Cell **107**(2): 173-182.

Vassar, R., B. D. Bennett, et al. (1999). "{Beta}-Secretase Cleavage of Alzheimer's Amyloid Precursor Protein by the Transmembrane Aspartic Protease BACE." Science **286**(5440): 735-741.

Verdile, G., R. N. Martins, et al. (2000). "Inhibiting Amyloid Precursor Protein C-terminal Cleavage Promotes an Interaction with Presenilin 1." J. Biol. Chem. **275**(27): 20794-20798.

Walsh, D. M., I. Klyubin, et al. (2002). "Naturally secreted oligomers of amyloid [beta] protein potently inhibit hippocampal long-term potentiation in vivo." Nature **416**(6880): 535-539.

Walsh, D. M., A. Lomakin, et al. (1997). "Amyloid beta -Protein Fibrillogenesis. DETECTION OF A PROTOFIBRILLAR INTERMEDIATE." J. Biol. Chem. **272**(35): 22364-22372.

Watanabe, N., T. Tomita, et al. (2005). "Pen-2 Is Incorporated into the {gamma}-Secretase Complex through Binding to Transmembrane Domain 4 of Presenilin 1." J. Biol. Chem. **280**(51): 41967-41975.

Weidemann, A., S. Eggert, et al. (2002). "A Novel <img src="http://pubs.acs.org/images/entities/epsiv.gif" border="0">-Cleavage within the Transmembrane Domain of the Alzheimer Amyloid Precursor Protein Demonstrates Homology with Notch Processing." Biochemistry **41**(8): 2825-2835.

Wolfe, M. S., M. Citron, et al. (1998). "A Substrate-Based Difluoro Ketone Selectively Inhibits Alzheimer's <img src="http://pubs.acs.org/images/gifchars/gamma.gif" border="0">-Secretase Activity." J. Med. Chem. **41**(1): 6-9.

Wolfe, M. S., W. Xia, et al. (1999). "Peptidomimetic Probes and Molecular Modeling Suggest That Alzheimer's <img src="http://pubs.acs.org/images/gifchars/gamma.gif" border="0">-Secretase Is an Intramembrane-Cleaving Aspartyl Protease." Biochemistry **38**(15): 4720-4727.

Wolfe, M. S., W. Xia, et al. (1999). "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and [gamma]-secretase activity." Nature **398**(6727): 513-517.

Wong, P. C., H. Zheng, et al. (1997). "Presenilin 1 is required for Notch1 DII1 expression in the paraxial mesoderm." Nature **387**(6630): 288-292.

Xia, W., W. J. Ray, et al. (2000). "Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid beta -protein generation." PNAS **97**(16): 9299-9304.

Xia, W., J. Zhang, et al. (1998). "Presenilin 1 Regulates the Processing of <img src="http://pubs.acs.org/images/gifchars/beta2.gif" border="0" align="middle">-Amyloid Precursor Protein C-Terminal Fragments and the Generation of Amyloid <img src="http://pubs.acs.org/images/gifchars/beta2.gif" border="0" align="middle">-Protein in Endoplasmic Reticulum and Golgi." Biochemistry **37**(47): 16465-16471.

Yan, R., M. J. Bienkowski, et al. (1999). "Membrane-anchored aspartyl protease with Alzheimer's disease [beta]-secretase activity." Nature **402**(6761): 533-537.

Yan, R., J. B. Munzner, et al. (2001). "BACE2 Functions as an Alternative alpha -Secretase in Cells." J. Biol. Chem. **276**(36): 34019-34027.

Yang, D. S., A. Tandon, et al. (2002). "Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins." J Biol Chem **277**(31): 28135-42.

Yu, G., F. S. Chen, et al. (1998). "The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin." Journal of Biological Chemistry **273**(26): 16470-16475.

Yu, G., M. Nishimura, et al. (2000). "Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing." Nature **407**(6800): 48-54.

Zhang, J., D. E. Kang, et al. (1998). "Subcellular Distribution and Turnover of Presenilins in Transfected Cells." J. Biol. Chem. **273**(20): 12436-12442.

Zhang, J. M., W. J. Ye, et al. (2002). "Proteolysis of chimeric beta-amyloid precursor proteins containing the Notch transmembrane domain yields amyloid beta-like peptides." Journal of Biological Chemistry **277**(17): 15069-15075.

Zhang, L., L. Song, et al. (2001). "Biochemical Characterization of the <img src="http://pubs.acs.org/images/gifchars/gamma.gif" border="0">-Secretase Activity That Produces <img src="http://pubs.acs.org/images/gifchars/beta2.gif" border="0" align="middle">-Amyloid Peptides." Biochemistry **40**(16): 5049-5055.

Zhao, G., G. Mao, et al. (2004). "Identification of a New Presenilin-dependent {zeta}-Cleavage Site within the Transmembrane Domain of Amyloid Precursor Protein." J. Biol. Chem. **279**(49): 50647-50650.

Appendix

"Dissertations are not finished; they are abandoned."
- Fred Brooks

This Appendix contains reprints of the following articles:

Shah S, Lee SF, Tabuchi K, Hao Y, Yu C, LaPlant Q, Ball H, Dann III C, Südhof T, Yu G. **Nicastrin Functions as a gamma-Secretase-Substrate Receptor.** Cell, 2005 Aug 12;122(3):435-447

Lee SF, **Shah S**, Yu C, Wigley WC, Li H, Lim M, Pedersen K, Han W, Thomas P, Lundkvist J, Hao YH, Yu G. **A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex.** J Biol Chem. 2004 Feb 6;279(6):4144-52.

Luo WJ, Wang H, Li H, Kim BS, **Shah S**, Lee HJ, Thinakaran G, Kim TW, Yu G, Xu H. **PEN-2 and APH-1 coordinately regulate proteolytic processing of presenilin 1.** J Biol Chem. 2003 Mar 7;278(10):7850-4.

Lee SF, **Shah S***, Li H, Yu C, Han W, Yu G. **Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-beta precursor protein and Notch.** J Biol Chem. 2002 Nov 22;277(47):45013-9**. *co-first author**.

Shah S, Yu G. **Viewpoint; SorLa: Sorting Out APP.** Molecular Interventions; In press.

This Appendix contains list of Figures:

This Appendix contains reprints of important protocols.

Ni-NTA for binding assays under conditions of co-expression in mammalian or insect cells.

1) Cells are washed 1X with. Add lysis buffer to cells on ice for 60 min. Ideal final concentration should be around 1-5 mg/ml.

Lysis Buffer (stored at 4◦C)
0.5% Triton X 100
50 mM Sodium Phosphate Buffer pH 7.2
150 mM NaCl
+ Protease Inhibitors (Roche EDTA free tabs, PMSF, Aprotinin, Leupeptinin)

2) Ni-NTA beads, wash beads in binding buffer (-detergent) and are then blocked overnight with 5mg/ml BSA. Add 0.01% thimerosal if stored for longer than 24hrs. Mix Ni-NTA beads with 10mM imidazole for 10 mins immediately before adding the beads to the lysate.

3) Lysed cells are spun at 14K rpm for 20mins and the lysate (supernatant) + ~ 30ul packed bead volume to ~ (500 to 1000ul of lysate) + 15mM imidazole final concentration are incubated 6 hrs to overnight for binding on a rotator at 4◦C.

4) After binding, spin tubes down @ 6K rpm for 5 min. Save lysate as flow through.

5) Wash 5X with 950ul wash buffer*, 5 min washes each @ 4◦C. Collect the beads by spinning tubes down @ 10K rpm for 3min.

Wash Buffer (stored at 4◦C)
0.5% Triton X 100
50 mM Sodium Phosphate Buffer pH 7.2
300 mM NaCl or 500mM NaCl
+ Protease Inhibitors (Roche EDTA free tabs, PMSF, Aprotinin, Leupeptinin)

*Several experiments were done in parallel using the two different salt concentrations for washes.

For Large Scale purification (for e.g. Expression in Insect Cells) a gradient salt wash of 150mM to 800mM for purification of Nicastrin or co-purification of Nicastrin/Substrate complex over Ni-NTA column was performed.

6) After washes, Elute Ni-NTA beads with 30ul of low pH (0.2% Triton X-100 in 100mM Glycine-HCL, pH 2.8) 3X, 5 min incubations. Neutralize the elution with ~10ul of (1M Trish HCl, pH 12.0).

Elution Buffer (stored at 4◦C)
0.2% Triton X 100
100mM Glycine-HCL, pH2.8

<u>Co-Immunopreciptation protocol for endogenous or coexpression binding assays of proteins of interest in mammalian or insect cells.</u>

Lysis Buffer (stored at 4◦C)
0.5% Triton X 100
50 mM Sodium Phosphate Buffer pH 7.2
150 mM NaCl
+ Protease Inhibitors (Roche EDTA free tabs, PMSF, Aprotinin, Leupeptinin)

1) Cells are washed 1X with. Add lysis buffer to lyse cells on ice for 60 min. Ideal final concentration should be around 1-5 mg/ml.

2) Spin 20 min @ 14K rpm to obtain insoluble pellet and lysate (supernatant).

3) Transfer supernatant (100-1000ul) into fresh tube and add appropriate amount of antibody (0.5-10ug purified antibody; or 0.5-10ul total antiserum)****, incubate with shaking at 4◦C overnight.

**** Note: Proper volume of supernatant and appropriate amount of antibody depend on number of factors, such as expression levels of the target proteins, affinity of the antibody, and technical consideration for western blotting etc. Appropriate conditions need to be tested experimentally. For APP-CTF U955 TCS lab antibody a 1:1000 or 1:2000 dilutions seems to work the best for IPing APP and APP fragments.

4) Protein-A-sparse beads are washed in lysis buffer (- detergent) and then blocked overnight with 5mg/ml BSA.

5) Add ~ 20 to 30ul pre-blocked protein-A-sparse and incubates with shaking at 4◦C for 4 to 6 hrs.

6) After incubation, spin down beads and save lysate as flow through.

7) Wash beads 5X with 950ul of wash buffer, 5mins each on 4◦C shaker. Collect beads by centrifugation at 6000 rpm for 5min.

Wash Buffer (stored at 4◦C)
0.5% Triton X 100
50 mM Sodium Phosphate Buffer pH 7.2
300 mM NaCl or 500mM NaCl
+ Protease Inhibitors (Roche EDTA free tabs, PMSF, Aprotinin, Leupeptinin)
*Several experiments were done in parallel using the two different salt concentrations for washes.

8) Elute proteins with ~30ul of pH (0.2% Triton X-100,100mM Glycine-HCl pH 2.8), 3X and neutralize the samples with ~10ul of (1M Tris pH 12.0) to neutralize.

Elution Buffer (stored at 4◦C)
0.2% Triton X 100
100mM Glycine-HCL, pH2.8

Phosphate (Sodium) buffer Chart

Stock solution A
2 M monobasic sodium phosphate, monohydrate (276g/L)
Stock solution B
2 M dibasic sodium phosphate (284 g/L).
Mixing an appropriate volume (**ml**) of **A** and **B** as shown in the table below and diluting to a total volume of 200 ml, a 1 M phosphate buffer of the required pH at room temperature.

A	B	pH
92.0	8.0	0.8
90.0	10.0	5.9
87.7	**12.3**	**6.0**
85.5	15.0	6.1
81.5	19.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	**31.5**	**6.5**
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	**61.0**	**7.0**
33.0	67.0	7.1
28.0	72.0	7.2

23.0	77.0	7.3
19.0	81.0	7.4
16.0	**84.0**	**7.5**
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8

Protocol to Purify C100FLAG and Formylated C100Flag

Purpose:

Purify large enough amount of immediate γ-secretase substrate C100FLAG & formylated C100FLAG to use for *in vitro* or binding assays.

Material Needed:

C100FLAG cDNA (pET21B) in Rossetta BL-21 or AG100A (DE3) cells.

Protocol:

1. Pick a colony of cells from LB AMP plate or from original glycerol stock. Inoculate 50 ml of LBA media, shake at 37◦C overnight

2. Prepare 2x 500ml LBA media, and add 10 ml overnight culture, shake at 37◦C 2 hours till OD 600 reaches above or close to 0.6 – 0.75.

3. Let it cool down to room temp. Add 50 ul 1M IPTG to a final concentration of 100uM. For formylated C100 add 20 ul actinonin (PDF inhibitor) to 2 ug/ml, shake at 37◦C (prefer to shake at RT) for 5 hours.

4. Spin cultures down at 4000 rpm for 10mins. Resuspend in 25 ml 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40 and Roche proteases, freeze at -80◦C.

5. Thaw the pellets and break cells with N2 bombing, 1500 psi for 15min at least 3X.

6. Spin at 35000 rpm for 1 hour in Ti45 rotor, save supernatant. If pellet remains large, resuspend in 10 ml Tris buffer with 1% NP40 and run through N2 par bomb or homogenizer again.

7. Spin at 35000 rpm for 1 hour to get supernatant. Combine supernatants, and dilutee with PBS to 3 x 50 ml tubes. Add 3x 0.2 ml anti-FLAG M2 slurry, rotate overnight at 4◦C.

8. Pass though a column, was column with 50 ml 0.1% NP-40 in PBS

9. Elute with 3x 0.5 ml 100 mM glycine pH 2.5, 1% NP-40 (add 0.04 ml 1 M Tris to neutralize).

Protocol for preparing electro-competent cells DH10B

1. Streak frozen cell stock on LB plate, 37◦C, O/N
2. Pick a single colony and inoculate into 50ml LB medium Shake at 37 C, O/N.
3. Inoculate 12 mo of O/N culture into 1 liter LB media (in 2 liter flasks, x 4). Shake at 37 C, 3hr (OD600=0.5-0.7)
4. Transfer cultures into 1 liter tubes (x4). Cfg. 3,500, 20 min, 4 C.
5. Discard supernatant completely.
6. Resuspend in 1 liter of ice cold H2O (x4). Cfg. 4,000, 20 min, 4 C.
7. Discard supernatants (to avoid losing pellets, leave supernatants little bit).
8. Resuspend in 500 ml of ice cold H2O (merge 4 tubes into 2 tubes) cfg. 4,200 rpm, 20 min 4 C.
9. Discard supernatants (to avoid losing pellets, leave supernatnats little bit).
10. Mix residual supernatants and pellets, and collect to Falcon 50 ml tubes (~8 tubes).
11. Cfg. 3,200 rpm, 20 min, 4 C.
12. Discard supernatants.
13. Add 10 ml of 10% glycerol in each tube, mix and assemble into 1 tube (finally ~30 ml).
14. Aliquot to epeendorf tube (on dry ice/ methanolor liquid nitrogen).
15. Store at -80 C.

All steps must be done on ice after step 6.

Protocol for DEAE-Dextran Dependent Transfection (transient transfection)

1. On day 0, plate 3 x 105 cells on 60 mm dishes in 3 ml normal medium
2. On day 1, mix 550 ul 2XTBS + 418 ul H2O + 22 ul DNA (0.1 mg/ml plasmid DNA, twice purified) + 110 ul DEAE dextran (5mg/ml, Pharmacia, MW = 5 x 10^5.)
3. Wash cells 2 times with 2 ml 1 XTBS.
4. Add 1 ml DNA/DEAE-dextran solution (from step #2) and incubate 30 min at 37◦C, 5% CO_2 .
5. Aspirate DNA/DEAE-dextran solution, being careful not to cross-contaminate dishes with DNA from other dishes.
6. Add 2 ml medium containing 100 uM chloroquine (from 10 mM stock freshly made in sterile water) and incubate 3 hours at 37 C.
7. Aspirate cholorquine medium and wash cells 2 times with 2 ml 1 XTBS.
8. Shock cells by incubating 4 minutes at room temperature in 2 ml 20% glycerol in DMEM (Can be made in advance, sterile filtered, stored 4◦C.)
9. Aspirate glycerol solution and gently wash cells 2 times with 2 ml 1XTBS.
10. Add 3 ml medium and incubate 48-60 hours at 37◦C.

2XTBS: 28.0 ml 5M NaCl
3.0 ml 1M KCl
1.0 ml 1M CaCl2
0.5 ml 1M MgCl2
4.5 ml 200mM Na2PO4, pH 7.4
20.0 ml 1M Tris-HCl, pH 7.9
Bring to 500 ml with ddH_2O
Steril Filter, store 4◦C

1XTBS: 1:1 dilution of 2XTBS, sterile filter.

For large scale transfections of COS, use 100 mm plates and scale up amounts by 3X.

Protocol for co-purification of C99-Flag and Nicastrin-V5-H6

Purpose:

Determine if Nicastrin directly binds to C99 and or any other γ-secretase substrates.

Determine stoichiometry or any other biochemical characteristics (e.g. binding affinity) of Nicastrin with its substrates.

Protocol

1. SF9 cells co-infected with NicV5H6 and C99FLAG
2. Membrane Preparation from insect cells
3. Extraction using 0.5% Triton X-100 (5ml membrane, 50 ml lysate)
4. Preclearing of lysate and incubation with M2 beads. (1 ml of M2 beads)
5. Extensive Wash (50ml, 300 mM NaCl 20 mM Imidazole)
6. Elution of M2 beads with FLAG Peptide. (200 ug/ml; 5ml)
7. Incubation of Eluted sample with Ni-NTA beads. (100ul)
8. Extensive Wash (5ml, 300 mM NaCl; 20 mM Imidazole)
9. Elution with low pH or high EDTA.

Controls

Pre-Incubate M2 beads with saturating levels of FLAG peptide and also during binding.

Pre-Incubate the Ni-NTA beads with high concentration of EDTA check pH of buffer.

Sf9 cells membrane of Nicastrin V5 H6 incubation with M2 beads. Sf9 cell membrane of C99-FLAG with Ni-NTA beads.

Sf9 cell membranes that are co-infected with Nicastrin V5 H6 and X-noninteracting protein FLAG or C99FLAG and X-noninteracting protein H6.

Protocol for Sf9 insect cells culture

Excell 420 media (JRH cat # 14420, ready to use withough any supplement. WARM TO ROOM TEMP before use.

Utilize good sterile techniques throughout.
Glassware has to be baked at 225◦C for 10 h.
Use glassware that is about two to three volume larger then the volume of media.
Incubate cells at 27-28◦C at 110-130 rpm.

1. Maintainance of cells.

For normal maintainance of cells, seed either 25 ml or 50 ml media to obtain 1.2 X 106 cells/ml. (Count the cells using the hemocytometer). Doubling time is about 24-30h. Do not let the culture exceed 8 x106 cells/ml. Therefore after 3 to 4 days, the cells have to be split and the process repeated again.

2. Amplificaton of cells for experiments

When you need a lot of cells, seed larger volume of media at 1 X 106 cells/ml. Grow until they are about 4 to 6 x 106 cells/ml and then you can use this stock to either expand again or set up for experiments.

3. Set up for experiments and infecting Sf9 cells

Seed cells at 1.5 X106 cells/ml, let the cells rest for overnight. Next day, only infect cells that are at 1.5 to 2.2 x 106 cells/ml with 1/100 X volume of virus. If you are using multiple virsuses, use 1/200X of each virus. Culture the cells for 44-48h.

4. Harvesting of cells.

Prior to harvesting, look at the cells under the scope, they should look plump but not lysed. If they are lysed, start all over again.
Harvest cells by spinning the cells at 800 to 1000xg, 10 min. (For most rotors, 2500 rpm, 10 min)

Protocol for Minipreping DNA using the alkaline lysis method.

1. If there are multiple tubes then you need to keep close track fo your clone number.

2. Transfer 1.3 ml overnight culture into microfuge tube.

3. Spin 13 K, 1 min. Remove sup.

4. Resuspend the bacteria pellet in 0.25 ml P1 buffer. Titurate.

5. Add 0.25 ml P2. Invert 5 times.

6. Add 0.25 ml P3. Invert 5 times.

7. Spin 13 K, 10 min. (May need to spin down the SDS precipitate in multiple new tubes.)

8. Prepare new microfuge tubes containing 0.7 ml isopropanol.

9. Once the sample is spun, KEEP the supernatant and add it to the isopropanol.

10. Invert 5 times and spin 13 K, 30 min.
    Make a note to know where the pellet will end up

11. Remove the sup.

12. Wash the pellet with 1 ml 70% ethanol.

13. Remove the sup. Leave to airdry upside down for 5 -10 min.

14. Resuspend the pellet in 40 ul H2O. DNA is good for restriction enzyme digest, sequencing, transfection, etc.

Preparation of buffers

Buffer compositions are given per litter of solution. Do not autoclave MOPS- or isopropanol-containing buffers; sterilize by filtration instead.

Buffer calculatons are based on Tris base adjusted to pH with HCL (Tris.Cl). If using Tris-HCL reagent, the quanitites used should be recalculated.

P1: Dissolve 6.06 g Tris base, 3.72 g Na2EDTA.2H2O in 800ml dH2O. Adjust the pH to 8.0 with HCL. Adjust the volume to 1 liter with dH2O. Add 100 mg RNase A per liter of P1.

P2: Dissolve 8.0 g NaOH pellets in 950 ml dH2O, 50 ml 20% SDS (w/v) solution. The final volume should be 1 liter.

P3: Dissolve 294.5g potassium acetate in 500 ml dH2O. Adjust the pH to 5.5 with glacial acetic acid (~110 ml). Adjust the volume to 1 liter with dH2O.

Preparation of LB medium

Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 ml dH2O. Adjust the pH to 7.0 with 1 N NaOH. Adjust the volume to 1 liter with dH2O. Sterilize by autoclaving.

Preparation of Coomassie Blue Stain: (for gels)

1) Combine 225 ml Methanol with 225 ml ddH2O.
2) Add 0.5 grams of Coomassie Blue.
3) Just before use, add 50 ml acetic acid to 10% final concentration.
4) Stain for 2 hours. Staining time can be shortened by microwaving; ~50 seconds at medium power.
5) After staining, pour off the stain into a bottle marked Used Stain. Stain can be re-used once, but fresh 10% acetic acid must be added).

Preparation of Coomassie Blue Destain: (for gels)

1) Combine 25 ml of Methanol with 437.5 ml ddH2O.
2) Just before use, add 37.5 ml acetic acid to 7.5% final concentration.
3) Destain, plus sponges (pieces of foam packing) for a couple of hours. It may be necessary to use more than one change of destain. Microwaving can be used in this stage also, but the details have not been determined.

Preparaton of Coomassie Blue Stain: (for membranes)

1) Combine 50 ml of Methanol with 50 ml of ddH2O.
2) Add 0.1 g of Coomassie Blue.
3) Stain by pouring a sufficient amount to cover the membrane in a petri dish.
4) Wash the fluid gentle back and forth over the membrane. Allow it to be immersed for one to two minutes.
5) Dispose of stain in a manner similar to that for the gel stain.

Preparation of Dialysis Tubing Prep

1) Cut tubing to appropriate lengths and place them in a large beaker.
2) Add enough 5% $NaHCO_3$ (add 50 grams per liter of ddH2O) to cover the tubing.
3) Bring to a rolling boil and allow it to boil for 15 minutes. The tubing will float so place a beaker of the next smaller size on top of the tubing.
4) Rinse the tubing with an excess of ddH2O.
5) Soak the tubing in 2 mM EDTA (add 10 ml 0.2 M to 1 liter ddH2O) for one hour.
6) Rinse again with an excess of ddH2O.
7) Store in 0.05% Azide (add 0.005 ml per liter of ddH2O).

Preparaton for pouring stacking gels

0.4mls Acrylamide
0.4mls 10x Stacking Buffer
40uls 10% SDS
3.12mls ddH20
2uls Temed
34uls APS

Preparaton to make 15% mini-gels

Important Due to the fact that Acrylamide is being used, it is extremely important to wear gloves throughout this procedure.
1) Measure out 5.152-ml ddH2O into a sterile 15-ml-tube.
2) Add 4.58-ml Acrylamide.
3) Next add 1.1-ml 10x Seperating Buffer.
4) Then add 110-ml 10% SDS.
5) Continue by adding 56-ml APS.
6) Finally, add 5.6-ml Temed.(Note: Temed is the polymerizing agent so the following steps must be done fairly quickly.)
7) Use a disposable pipet, homogenize the solution being careful not to cause any

bubbles.
8) Then use the same pipet to fill the glass leaving about 1 inch at the top.
9) Finally, add 0.5 inch of H2O-saturated butanol on top of the gel. Note: Leave the unused portion of the solution in the 15-ml tube. Use this to determine if your gel is ready to use (when the tube is polymerized, so is your gel).

Protocol to Strip Western Blot Membranes

Procedure

1. Put the membrane in PBS or TBS with 7ul/ml B-mercaptoetanol and 2%SDS for 30' at RT in agitation
2. Wash the membrane for 30' in PBS or TBS.
3. Put the membrane in blocking buffer

Protocol for preparing solutions and chemicals that are commonly used in the lab.

Acetic acid (MW 60.05)

Glacial acetic acid is 99.6% (w/v) acetic acid, and is 17.4 M.

1 M	Add 57.5 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
0.05 N	Add 2.87 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
0.9 M	Add 54 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
7% (w/v)	Add 70 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
45% (w/v)	Add 450 ml of glacial acetic acid to 500 ml of water and then make to 1 liter with water.

Acetic Acid/Butanol/Water (15:60:25)

Combine 150 ml of glacial acetic acid, 600 ml of nbutanol and 250 ml of water.

Acid alcohol

Add 1.0 ml of concentrated HCl to 100 ml of 70% (v/v) ethyl alcohol.

Acrylamide Solutions

Acrylamide solutions for PAGE are given as total concentration of acrylamide (acrylamide + bisacrylamide) and the amount of cross linker (bisacrylamide). This is listed as the T:C ratio. For example, a 10% gel (10%T:5%C) would contain a total of 10 g rams of acrylamide per 100 ml, and would be composed of 5 grams of acrylamide and 5 grams of bisacrylamide. Usually, a stock solution of 30% acrylamide is produced containing 0.8% bis-acrylamide. Many investigators use 30 grams of acrylamide plus 0.8 gram s of bis-acrylamide per 100 ml of water, but 29.2 grams of acrylamide plus 0.8 grams of bis would be technically correct. In practice, it makes little difference since the gels are diluted to 10% or less. The 30% stock solution is filtered through a 0.45 µ filter and stored at 4 ° C in the dark. For use, the stock solution is diluted with an appropriate buffer (usually a 2X Tris-HCl). The stock solution is stable for about one month. Discard after this period.

Acrylamides in their monomeric form are neurotoxic. Polymerize all acrylamide solutions prior to disposal.

SDS,β-mercaptoethanol and a tracker dye (bromophenol blue) are added at various points. Refer to Chapter 4 for more complete details.

Alkaline Distilled Water

Add one pellet of NaOH to 1 liter of distilled water.

Ammonium acetate (MW 77.08)

0.1 M	Add 7.708 grams of Ammonium acetate to a final volume of 1 liter of water.

Ammonium persulfate (MW 228.2)

10% (w/v)	Dissolve 1.0 grams of ammonium persulfate to a final volume of 10 ml with water. Mix fresh, prior to use as a catalyst for PAGE. Normally, about 50 µ l of ammonium persulfate is added to each 15 of gel solution for polymerization.Dissolve 0.13 grams o f Alcian Blue 8GX (Sigma # A-2899) in 100 ml of water.

Ammonium sulfate (MW 132.14)

2% (w/v)	Add 2 grams of ammonium sulfate to a final volume of 100 ml water.
4.1M(sat.) 0.001 M	Dissolve 542 grams of ammonium sulfate to a final volume of 1 liter.

Ascorbic acid (MW 176.12)

2 mM	Dissolve 35.2 mg of ascorbic acid to a final volume of 100 ml with water.

ATP (Adenosine triphosphate, MW 507.21)

5 mM — Dissolve 254 mg of ATP to a final volume of 100 ml with water or buffer.Dissolve 35.2 mg of ascorbic acid to a final volume of 100 ml with water.

Benzoic acid (MW 122.12)

8 mM — Dissolve 98 mg of benzoic acid to a final volume of 100 ml with water or buffer.

Bis-acrylamide (N,N'-Methylene-bis-acrylamide)

Cross linker for acrylamide gels. Refer to Acrylamide solutions or Chapter Four for more details.

Bovine Serum Albumin (BSA)

There are many grades of BSA available and care should be taken when using this protein. For routine protein concentration standards, a 96-99% pure fraction (Sigma # A 2153) may be used. For tissue culture, RIA, or molecular weight standardization, BSA sh ould be optained which is extracted and purified specifically for that purpose.

1% (w/v) — Dissolve 0.5 grams of BSA to a final volume of 50 ml in water or buffer.

Bradford Protein Assay

This procedure uses an absorbance shift in an acidic Coomasie Blue solution. It is commercially available from Pierce Chemical Company, Rockford, Illinois as Protein Assay Reagent, Cat. # 23200. It contains methanol and solubilizing agents and is very reliable.

If you wish to make your own, dissolve 100 mg of Coomasie Brilliant Blue G-250 in 50 ml of 95% ethanol. Add 100 ml of 85% phosphoric acid, and bring to a final volume of 1 liter with distilled water.

Phosphoric acid is extremely corrosive. Handle with care.

Bromophenol blue (Sodium salt, MW 692.0)

0.001 (w/v) — Dissolve 1 mg of Bromophenol blue, sodium salt (Sigma # B7021) to a final volume of 100 ml with either water or buffer.

n-Butanol (C_4H_9OH MW 74.12)

Density = 0.8098 grams/ml

1.1 M — The butanol can be weighed (81.5 grams) or measured volumetrically by using the density. That is, 81.5 grams ÷ 0.8098 grams/ml or 100.7 ml of n-butanol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Calcium chloride (MW 110.99)

0.0033 M	Dissolve 0.522 grams of calcium acetate to a final volume of 1 liter with water or buffer.

Calcium chloride (MW 110.99)

0.001 M	Dissolve 0.111 grams of anhydrous calcium chloride to a final volume of 1 liter with water or buffer.
0.08 M	Dissolve 8.879 grams of anhydrous calcium chloride to a final volume of 1 liter with water or buffer.
2% (w/v)	Dissolve 2 grams of anhydrous calcium chloride to a final volume of 100 ml with water or buffer.

cAMP (Adenosine monophosphate, cyclic MW 329.22)

0.001 M (1mM)	Dissolve 33 mg of cAMP to a final volume of 100 ml with water, buffer or media.

Citric acid ($H_3C_6H_5O_7 \cdot H_2O$ MW 210.14)

0.1 M	Dissolve 21.01 grams of citric acid to a final volume of 1 liter.

Citrate buffer (Sodium phosphate-Citrate buffer)

PH 4.8	Add 493 ml of 0.2 M Na_2HPO_4 to 507 ml of 0.1 M citric acid.
PH 3.6	Add 322 ml of 0.2 M Na_2HPO_4 to 678 ml of 0.1 M citric acid.
PH 4.2	Add 414 ml of 0.2 M Na_2HPO_4 to 586 ml of 0.1 M citric acid.
PH 5.4	Add 557.5 ml of 0.2 M Na_2HPO_4 to 442.6 ml of 0.1 M citric acid.
PH 6.0	Add 631.5 ml of 0.2 M Na_2HPO_4 to 368.5 ml of 0.1 M citric acid.
PH 6.6	Add 727.5 ml of 0.2 M Na_2HPO_4 to 272.5 ml of 0.1 M citric acid.
PH 7.2	Add 869.5 ml of 0.2 M Na_2HPO_4 to 130.5 ml of 0.1 M citric acid.
PH 7.8	Add 957.5 ml of 0.2 M Na_2HPO_4 to 42.5 ml of 0.1 M citric acid.

Coomasie blue (Coomasie Brilliant Blue R250)

0.25% (w/v)0.001 M	Dissolve 2.50 grams of Coomasie Brilliant Blue R250 to a final volume of 1 liter with 20% (w/v) trichloroacetic acid (TCA). Some investigators use a 0.25% solution of Coomasie Blue in methanol-water-glacial acetic acid (5-5-1).

Copper sulfate ($CuSO_4 \cdot 5H_2O$ MW 249.68)

0.5% (w/v) Dissolve 0.13 grams of Alcian Blue 8GX (Sigma # A-2899) in 100 ml of water.

0.5% (w/v)

Dissolve 0.5 grams of copper sulfate to a final volume of 100 ml with water.

Copper tartrate/carbonate (CTC)

Dissolve 0.5 grams of copper sulfate and 1.0 gram of potassium sodium tartrate to a final volume of 100 ml with water. Combine 1.0 ml of this solution with 50 ml of 2% Na_2CO_3 in 0.1 N NaOH. Must be made fresh, prior to use. Stock solutions are stable.

Crystal Violet

Dissolve 0.1 grams of crystal violet and 0.25 ml of glacial acetic acid to a final volume of 100 ml with water.

Dithiothreitol (MW 154.3)

0.01 M Dissolve 154 mg of dithiothreitol to a final volume of 100 ml with water or buffer. Dithiothreitol is available from Sigma Chemical Co., St. Louis, Cat # D0632. Dithioerythritol may be substituted.

EDTA (Ethylenediaminetetraacetic acid MW 292.24)

1 M Dissolve 292.24 grams of EDTA, free acid to a final volume of 1 liter. If the more soluble disodium salt of EDTA is used, adjust the weight accordingly. The pH can be adjusted with acetic acid or NaOH. For corresponding concentration dilutions, multiply the weight in grams by the desired molarity. For exam ple, for 10 mM EDTA, multiply 292.24 X 0.010 to obtain 2.92 grams of EDTA per liter.

EGTA (Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid MW 380.4)

1 mM dissolve 380 mg of EGTA to a final volume of 1 liter with water or buffer.

Ethanol (C_2H_5OH MW 46.07)

Density = 0.7893 gm/ml

50-95% (v/v) Since 95% ethyl alcohol is less expensive and easier to store than absolute, these dilutions should be made with 95% ethyl alcohol. Unless otherwise stated, denatured alcohol works as well as the more expensive non-denatured. A simple way to make the % so lution is to use the appropriate amount of 95% ethanol and dilute to 950 ml instead of 1 liter. For example, to make a 50% (v/v) solution, measure out 500 ml of 95% ethyl alcohol and dilute to a final volume of 950 ml with water. For a 70% solution, meas ure 700 ml of ethyl alcohol and dilute to 950 ml with water. Absolute ethanol should be used directly as 100% ethanol. It is important for histology that this be truly 100%. Since it is hydroscopic (it

absorbs water from the air), do not assume it is abso lute unless it is sealed or treated to ensure no water. To test, add a drop to a sample of xylol. If any cloudiness occurs, the alcohol is not absolute.

8.5 M The ethanol can be weighed (391.6 grams of absolute, 412.2 grams of 95% (v/v) or measured volumetrically by using the density. That is, 391.6 grams ÷0.7893 grams/ml or 496.1 ml of absolute ethanol. Using 95%, 412.2 grams ÷0.7893 grams /ml or 522.2 ml. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Giemsa stain

Prepare a stock solution by dissolving 3.8 grams of giemsa powder in 25 ml of glycerin. Heat gently with stirring for about 2 hours at 60 ° C. Cool and add 75 ml of methanol (neutral, acetone free).
For a working solution, dilute the stock solution 1/10 with water before use.
For chromosome banding, combine 5.0 ml of stock Giemsa, 3.0 ml of absolute methanol, 3.0 ml of 0.1 M citric acid and 89 ml of distilled water. Adjust the pH of the solution to 6.6 with Na_2HPO_4.

Glutaraldehyde (GTA)

5 % GTA is usually supplied as a 25% or 50% (w/v) solution. It is used for electron microscope fixation as a 5% solution in a buffer. For routine use, add 20 ml of 25% GTA to 80 ml of 0.2 M sodium cacodylate buffer, pH 7.4.

Glycerol (MW 92.09)

10% (v/v) To 10 ml of glycerol (glycerine) add enough water to make a final volume of 100 ml.

8 M Weigh 73.67 grams of glycerol and add to a final volume of 100 ml. Alternatively, measure 499.1 ml of glycerol and make to a final volume of 1 liter (the density of glycerol at room temperature is 1.476) with water or buffer.
For 8 M glycerol in MT buffer, make a 2X MT buffer for use as the diluent.

Glycine (MW 75.07)

0.192 M Dissolve 1.44 grams of Glycine to a final volume of 100 ml with water or buffer.

Hydrochloric Acid (HCl MW 36.46)

Concentrated HCl has a molarity of approximately 11.6.
HCl is a gas, which is soluble in water and which comes in the form of concentrated reagent grade HCl. This solution is approximately 36-38% (w/v) HCl. To make a 1 N solution, add 86 ml of concentrated HCl to 800 ml of water and dilute to a final volume of 1 liter. For 0.1 N, dilute the 1 N by a factor of 10.

For % solutions, note that liquid HCl is only 38% HCl, thus a 1% solution would require 2.6 ml of concentrated HCl (1/.38) per final volume of 100 ml.

Magnesium chloride ($MgCl_2$ MW 95.23)

1 mM	Dissolve 95.2 mg of magnesium chloride per final volume of 1 liter.
4 mM	Dissolve 0.381 grams of magnesium chloride per final volume of 1 liter.
10 mM	Dissolve 0.952 grams of magnesium chloride per final volume of 1 liter.
0.1 M	Dissolve 9.523 grams of magnesium chloride per final volume of 1 liter.

Note: A single stock solution of 1 M $MgCl_2$ can be mixed by dissolving 95.23 grams of magnesium chloride to a final concentration of 1 liter with water, and all dilutions made appropriately from this stock solution.

Magnesium sulfate ($MgSO_4$ MW 120.39)

5% (w/v)	Dissolve 5.0 grams of magnesium sulfate to a final volume of 100 ml with water or buffer.
0.5 M	Density = 1.2 grams/ml. Use either 3.91 grams OR 3.26 ml of mercaptoethanol in a final volume of 100 ml of water or buffer.
5% (w/v)	Use 5.0 grams or 4.167 ml in a final volume of 100 ml of water or buffer.

MES (2-(N-Morphilino)ethanesulfonic acid MW 195.2)

0.1 M	Dissolve 1.952 grams of MES to a final volume of 100 ml with water or buffer.

Methanol (CH_3OH MW 32.04)

Density = 0.7914 grams/ml

22 M	The methanol can be weighed (704.9 grams) or measured volumetrically by using the density. That is, 704.9 grams ÷ 0.7914 grams/ml or 890.7 ml of methyl alcohol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Methanol/Acetic Acid (for fixing proteins in acrylamide gels)

45%:12%	Add 120 ml of glacial acetic acid to 450 ml of methanol and dilute to a final volume of 1 liter with water.

Methanol/Acetic Acid (for destaining or fixing proteins in acrylamide gels)

5%:7%	Add 70 ml of glacial acetic acid to 50 ml of methanol and dilute to a final volume of 1 liter with water.

Phenazine methosulfate (PMS MW 306.34)

Mutagen and irritant.

0.033% (v/v) Add 33 µl of phenazine methosulfate to 90 ml of water or buffer and make up to 100 ml final volume. Must be made immediately prior to use.

Phenol mixture

Combine 555 ml of aqueous phenol (or 500 grams of phenol crystals plus 55 ml of water) with 70 ml of mcresol. Add 0.5 grams of 8-hydroxyquinoline.

Phenol will cause severe burns and readily dissolves all plastic and rubber compounds. Use extreme caution when handling this compound.

Phosphate buffered saline (PBS)

Mix 100 ml Ca++/Mg++ fr ee 10X PBSA with 800 ml of distilled water. Separately, dissolve 0.1 gram of magnesium chloride and 0.1 gram of anhydrous calcium chloride to a final volume of 100 ml with water. With constant stirring, slowly add the magnesium/calcium chloride solution t o the diluted PBSA. If a precipitate forms, start over, and add slower with continuous stirring.

Ca++/Mg++ free Phos phate buffered saline - 10X (10X PBSA)

Dissolve 80 grams of NaCl, 2.0 grams of KCl, 15.0 grams of Dibasic sodium phosphate and 2.0 grams of Monobasic potassium phosphate in 1 liter of distilled water. This makes a 10X solution of Ca++/Mg++ *free phosphate buffered saline. Dilute 1:10 prior to use. Store in a refrige rator.*

Potassium chloride (KCl MW 74.55)

1 M Dissolve 74.55 grams of KCl to a final volume of 1 liter with water or buffer. For other concentrations, multiply the weight by the required molarity. For example, for 0.150 M (150 mM), use 0.150 X 74.55, or 11.183 grams of KCl in 1 liter of water or buffer. Use half as much to obtain 0.075 M for karyotyping.

Potassium phosphate, monobasic (KH_2PO_4 MW 136.09)

0.01M Dissolve 1.36 grams of monobasic potassium phosphate to a final volume of 1 liter with water.

Potassium phosphate, dibasic (K_2HPO_4 MW 174)

0.01 M Dissolve 1.74 grams of dibasic potassium phosphate to a final volume of 1 liter with water.

Potassium phosphate buffer

0.01M pH 7.4 — Prepare 500 ml of 0.01 M K_2HPO_4 and 500 ml of 0.01 M KH_2PO_4 Place the K_2HPO_4 onto a magnetic stirrer and insert a pH electrode. Ad d the KH_2PO_4 slowly to adjust the pH to 7.4.

Potassium hydroxide (KOH MW 56.10)

0.5 N — Dissolve 28.05 grams of KOH to a final volume of 1 liter with water.

10% (w/v) — Dissolve 10 grams of KOH to a final volume of 100 ml with water. Store in a plastic container.

Saline (NaCl)

0.85% (w/v) — Saline refers to a solution of NaCl, with the most common usage for that which is isotonic to mammalian blood cells, notable a 0.85% or 0.9% solution. To mix, dissolve 8.5 grams of NaCl to a final volume of 1 liter with water.

SDS

Refer to Sodium lauryl sulfate.

1X SDS-Electrophoresis Running Buffer

Dilute 5X Tris-Glycine buffer to 1X and add 1.0 gram of SDS per liter of 1X Tris-Glycine. The pH should be 8.3 after dilution.

2X SDS Sample Buffer

Dissolve 1.52 grams of Tris base, 2.0 grams of SDS, 20 ml of glycerin, 2.0 ml of -mercaptoethanol and 1 mg of bromophenol blue to a final volume of 100 ml with water.

Silver nitrate solution (for electrophoresis staining)

Dissolve 0.15 grams of NaOH in 150 ml of water. Add 3.5 ml of concentrated NH_4OH and bring to a volume of 200 ml. Separately, dissolve 2.0 grams of silver nitrate in a final volume of 10 ml. With constant stirring, add 8.0 ml of the silver nitrate to the 200 ml of $NaOH/NH_4OH$.

This solution should be prepared immediately prior to use, and used within 30 minutes.

Dispose of this solution with copious flushing. It becomes explosive upon drying.

Sodium acetate (MW 82.04)

1 M — Dissolve 82.04 grams of sodium acetate to a final volume of 1 liter with water or buffer.

0.02 M — Dissolve 1.64 grams of sodium acetate to a final volume of 1 liter with water or buffer.

Sodium acetate buffer

1 M pH 5.7 To 925 ml of 1 M sodium acetate, add 75 ml of 1 M acetic acid.

Sodium bicarbonate ($NaHCO_3$ MW 84.0)

0.1 M	Dissolve 0.84 grams of $NaHCO_3$ to a final volume of 100 ml with water or buffer.

Sodium chloride (MW 58.44)

M	For a molar solution of sodium chloride, dissolve 58.44 grams of NaCl to a final volume of 1 liter with water or buffer. For corresponding dilutions, multiply the weight by the molarity required. For example, for 0.05 M, multiply 58.44 by 0.05 or 2.92 grams/liter.
%	For % solutions, they are invariably w/v. For a 1 % (w/v) solution, dissolve 1.0 gram of NaCl to a final volume of 100 ml with water or buffer. Multiply the weight by a corresponding change in % for other concentrations.
200,300,400 mOsM	Osmoles for NaCl are calculated as twice the molar concentration. Thus, a 200 mOsM solution would be .100 M NaCl. Likewise, 300 mOsM would be .150 M and 400 mOsM would be .200 M NaCl.

Sodium citrate (MW 294.10)

0.09 M	Dissolve 2.65 grams of sodium citrate to a final concentration of 100 ml with water.

Sodium lauryl sulfate (SDS or SLS MW 288.38)

0.1% (w/v)	Dissolve 0.1 grams of SDS to a final volume of 100 ml with water or buffer. Mix by gentle stirring, do not shake.
10% (w/v)	Dissolve 10 grams of SDS to a final volume of 100 ml with water.

SDS should not be inhaled in its powder form. When weighing, use a mask, or better, a hood.

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$ MW 137.99)

1 M	Dissolve 14.01 grams of sodium perchlorate to a final volume of 100 ml with water or buffer.
0.01 M	Dissolve 1.38 grams of monobasic sodium phosphate to a final volume of 1 liter.

Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$ MW 268.07)

1 M	Dissolve 268.07 grams of dibasic sodium phosphate to a final volume of 1 liter.
0.2 M	Dissolve 53.61 grams of dibasic sodium phosphate to a final volume of 1 liter.
0.01 M	Dissolve 2.68 grams of dibasic sodium phosphate to a final volume of 1 liter.

Sodium phosphate buffer

These are the most common buffers used in biology. They are produced by adding equimolar solutions of KH_2PO_4 and Na_2HPO_4. Equal volumes of the two will yield a pH of 7.0, while sodium phosphate will increase the pH. Increased volumes of potassium phosphate will decrease th e pH. The pH can be adjusted from 5.4 to 8.2.

If a pH of 7.0-8.2 is desired, start with 500 ml of sodium phosphate and add potassium phosphate while stirring and monitoring the pH with a pH meter until the desired pH is reached.

If a pH of 5.4-7.0 is desired, start with 500 ml of potassium phosphate and add sodium phosphate until the desired pH is reached.

Typically, the molarity of the buffer will range from 0.01 to 0.1 M. Use the appropriate molarity of KH_2PO_4 and Na_2HPO_4. That is, if 0.05 M buffer is desired, use 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4 as directed above.

Sorbitol (MW 182.17)

0.33 M	Dissolve 60.12 grams of sorbitol to a final volume of 1 liter with water or buffer.

Sucrose (MW 342.3)

1.0 M	Dissolve 34.2 grams of sucrose to a final volume of 100 ml with water or buffer. For other molarities, multiply the weight by the required molar concentration. For example, for 0.25 M sucrose, weight 34.2 x 0.25 or 8.55 grams to a final volume of 100 ml.
40% (w/v)	Dissolve 40 grams of sucrose to a final volume of 100 ml with water or buffer. Dilute this solution for lower percent requirements. If using for sucrose density gradients, the sucrose should have 0.1 ml of diethylpyrocarbonate added, the solution brou ght to a boil for 3-5 minutes and cooled before use. This will eliminate RNAase, which would otherwise be a contaminant of the solution. Store all sucrose solutions in a refrigerator

TEMED (N,N,N',N'-tetramethylethylenediamine)

Catalyst for PAGE. Use directly and add 10 l TEMED per 15 ml of gel solution.

Trichloroacetic acid (TCA CCl_3COOH MW 163.4)

Extremely caustic acid. Handle with care.

72% (w/v) Dissolve 72 grams of TCA to a final volume of 100 ml. TCA is hydroscopic and will readily absorb water. The solid crystals will become liquid if the stock bottle is placed in warm water, with a loose cap (melting point 57-58 ° C. It is easier to handle as a liquid. Storage of solutions greater than 30% (w/v) are not recommended as decomposition is rapid. Therefore these solutions should be made as needed.

Tris buffer

There are many variations on the basic Tris-HCl buffer combination, most of which are commercially available. Solutions with EDTA are known as TE buffers, while solutions with EDTA and acetic acid are known as TAE buffers. The terminology varies with the author, with Tris buffer being used to mean Tris-HCl solutions. Sigma Chemical Co., St. Louis, carries a full line of the buffers marketed under the tradename of Trizma (base and HCl).
The basic buffer is a combination of Tris (tris(hydroxymethyl)aminomethane) and HCl acid. These are sometimes referred to as Tris-base and Tris-HCl solutions. Tris buffers should not be used below a pH of 7.2 or above a pH of 9.0. Tris buffers are also ex tremely temperature senstive. Directions are given for room temperature (25 ° C). The pH will decrease approximately 0.028 units for each degree decrease in temperature.

1 M Dissolve 121 grams of Tris in 800 ml of distilled water. Adjust the pH with concentrated HCl. Dilute to a final volume of 1 liter. Lower required molarities can be diluted from this stock or mixed as combinations of lower molarities of Tris and HCl. It is important to measure the pH at the temperature an d molarity that will be used in the final analysis.

Trypan blue

0.2 % (w/v) Dissolve 0.2 grams of trypan blue to a final volume of 100 ml with water.

Trypsin

0.25% 0.25% Dissolve 0.25 grams of crude trypsin in PBSA to a final volume of 100 ml. Cold sterilize by filtration.
Alternatively, purchase pre-diluted crude trypsin, sold as 1:250 which is pre-sterilized as well.

Note: When using trypsin for tissue disaggregation, it must be subsequently inhibited by the use of serum in the culture media, or by the addition of soya bean trypsin inhibitor.

Tween 20 or 80 (Polyoxyethylene sorbitan mono-oleate)

1% Add 1.0 ml of Tween to 90 ml of water. Mix and dilute to a final volume

(v/v) of 100 ml with water. Note that Tween is extremely viscous and care must be taken to accurately pipette 1.0 ml. Wipe the outside of the pipette before dispensing.

Urea (MW 60.06)

2.5 M	Dissolve 15.02 grams of urea to a final volume of 100 ml with water or buffer.
10 M	Dissolve 60.06 grams of urea to a final volume of 100 ml with water or buffer.
14 M	Dissolve 84.08 grams of urea to a final volume of 100 ml with water or buffer.

Protocol for in vitro γ-secretase activity assay.

1. Grow cells. Wash cells in PBS. Use right away or store the plate at – 80 C.
2. Scrape and lyse cells in 50 mM Pipes, pH 7.4, 5 mM MgCl2, 150 mM KCl, 1 Roche protesase inhibitor tablet/ 10 ml buffer.
3. Parr bomb, 600 psi, 10 min. 4X
4. Spin the lysate at 800xg, 10 min.
5. Spin the sup at 100,000xg, 1 hr.
6. Resuspend the membrane particulate in the same lysis buffer, freexe in liq N2 and store at -80C.
7. Determin protein concentration of the resuspended membrane.
8. For γ-secretase activity assay, incubate the membrane at 3 mg/ml with 0.25% CHAPSO. Agitate 1h, 4C.
9. Spin 13K, 20 min to obtain the solubilized ditergent supernatant.
10. Set up tubes for assay @ 37◦C

58 ul solubilized detergent sample
+ 2 ul Notch 99 Flag H6 (purified from Sf9 cells, 0.3 mg/ml stock)

Remove 15 ul at 0, 2, 5 h and add 5 ul RIPA stop buffer 95 C, 10 min.

Tap spin

Load 5 ul of the supernatant on 15% gel.

Run the front off to get maximum separation between the 20kDa and 15 kDa protein standard.

Probe the blot with 1/2000X mouse anti-Flag.

4X RIPA

300 mM NaCl
3% NP-40
1.5% deoxycholate
0.3% SDS
150 mM Tris, pH 8
600 mM DTT
30% sucrose

Protocol for Blue Native Gel Electrophoresis

Stock solutions

49.5%T, 3%C Acrylamide
24 g acrylamide, 0.75 g bisacrylamide / 50 ml H_2O
Store at RT

3 x Gel buffer
150 mM BisTris-HCl, 1.5 M 6-amino-caproic acid, pH 7.0
Adjust pH to 7.0 with HCl at 4°C
Store at 4°C

75% (w/v) Glycerol
Store at 4°C

10 x Cathode buffer
0.5 M Tricine, 150 mM BisTris
No need to adjust pH
Store at 4°C

5 x Anode buffer
0.25 M BisTris-HCl, pH 7.0
Adjust pH to 7.0 with HCl at 4°C
Store at 4°C

2 x BisTrisACA
200 mM BisTris-HCl, 1 M 6-amino-caproic acid, pH 7.0
Adjust pH to 7.0 with HCl at 4°C
Store at 4°C

50BTH40G
50 mM BisTris-HCl, 40% (w/v) Glycerol, pH 7.0
Adjust pH to 7.0 with HCl at 4°C
Store at 4°C

Working solutions

1xCathode (-dye), 1x Anode buffer
Store at 4°C

1xCathode (+dye)
0.01% Serva G in 1xCathode buffer

It takes a while to dissolve ServaG.
Store at 4°C

BN-sample buffer

Serva G	50 mg
2 x BisTrisACA	500 ul
75% Sucrose	400 ul
H_2O	100 ul

Store at -20°C

Solubilization buffer

50BTH40G	100 ul
10% Detergent	40 ul
H_2O	60 ul

Detergent: TX-100, dodecyl-maltoside, digitonin or SDS

Water soluble digitonin should be used.

If you don't get water-soluble digitonin, purify as described in Mori et al. (1999) J Cell Biol. 146, 45-55. We've also tried 2X crystalized digitonin with essentially the same results.

Note: the original method uses ACA in the solubilization buffer.

5-13.5% gradient blue native gel

Mini gel with 0.75 mm spacer

	Sample (4%) for 2 gels	Separation (5%)	Separation (13.5%)
49.5% Acrylamide	0.242 ml	0.212 ml	0.573 ml
3 x Gel buffer	1.0 ml	0.7 ml	0.7 ml
75% Glycerol	0	0.14 ml	0.56 ml
H_2O	1.72 ml	1.028 ml	0.247 ml
TEMED	6 ul	2 ul	2 ul

5% AP	32 ul	11 ul	11 ul
Pour into a gradient maker		1.88 ml	1.8 ml
Total	3.0 ml	2.1 ml	2.1 ml

Sample gel height should be ~0.7 cm

Make gel one day before use and store at 4°C

Sample preparation

Wash thylakoids with 0.33 M Sorbitol, 50 mM BisTris-HCl, pH 7.0
Resuspend in 25 mM BisTris-HCl, 20% Glycerol, pH 7.0
Adjust [chl] to 2.0 mg/ml (or resuspend thylakoids in the resuspension buffer to 2.0 mg chl/ml)
Add one volume of solubilization buffer, gently vortexing after each drop
Agitate for 30 min (DM, TX-100), 30-60 min (Digitonin) at 0 to 4°C
Spin at ~100,000 g for 30 min
Transfer the supernatant to a new tube
Add 1/10 vol of BN sample buffer
Mix gently
Load 5.5 ul onto BN-gel

Protein standard

	Apoferritin +BSA	β-amylase +β-lactoglobulin
5 mg/ml protein stock	7.5 ul each	10.0 ul (amylase) 5.0 ul (lactoglobulin)
Digitonin Solubilization buffer	15.0 ul	15.0 ul
BN-sample buffer	3.0 ul	3.0 ul

Protein stocks in 20% glycerol, 25 mM BisTrsi-HCl, pH7.0

Mix gently
Load 5.5 ul

Protein marker	Mr (kDa)
Ferritin	880, 440
β-amylase	200
BSA	132, 66
β-lactoglobulin	35

Thyroglobulin (670kD) migrates at position close to ferritin dimer (880kD).

Electrophoresis

Set a BN gel in in the cold room (2-4 C°C in the cathode tank)
Load samples
Run at constant voltage of 100-200 V, 3-6 hrs.

For immunoblotting:

Exchange the cathode buffer (+dye) to cathode buffer lacking the dye after top 1/2 ~2/3 of the gel is covered with dye; then run until the free dye has run off of the bottom of the gel (~2 hrs). The gel should look like this:

Immunoblotting

Transfer buffer
    25 mM Tris, 192 mM Glycine, 20% Methanol
    Use room temperature transfer buffer to kill peroxidase-like activities.

Incubate BN-gel in 0.1% SDS, Transfer buffer for ~10 min at RT
    0.5 ml 10% SDS + 50 ml Transfer buffer
Assemble blotting sandwich with PVDF membrane
    PVDF membrane must be wet in methanol before equilibration in transfer buffer
Set in a blotter and run at constant voltage of 50 V for 45 min. - no ice in the chamber.
Stain the blot with 0.1% PonceauS, 5% acetic acid
    It's a little hard to see marker proteins.

Supplemental Chapter

Ongoing experiments that further address aspects of Nicastrin interaction with γ-secretase substrates

Attributions: In this chapter, I made a significant intellectual and hands on contribution. The aim of the following experiments were to further build and elucidate the mechanisms that the γ-secretase complex employs in recognizing and cleaving a hetrogenous set of ectodomain shed group I transmembrane proteins. I took a leading role on a majority of questions and problems associated with understanding the major role of Nicastrin in the γ-secretase complex. Some experiments were performed collaboratively. I received help from rotation students, advice and guidance from Dr. Thomas Südhof and Dr. Gang Yu in sub group meetings regarding these projects. Beyond this, it is difficult and may be inaccurate to attribute each aspect of the work individually except to acknowledge all past and present members of Dr. Gang Yu's lab as well as students who rotated through our lab that I worked very closely with.

Publications: Data in this chapter has not been published.

Abstract

This thesis spans a period of contribuitions that addresses the true identity of the γ-secretase complex as well as elucidates an aspect of its working mechanism. It established the role of nicastrin as a sensor for amino-terminal fragments of the substrates. It provides for the first time an explanation for why transmembrane proteins only become substrates for the complex after their ectodomain is shed. Furthermore, the work introduces novel and innovative approaches that may be used to further analyze and understand the biochemistry of the γ-secretase complex. We proposed that γ-secretase evolved in a unique and elegant convergence whereby the substrate recognition function of an aminopeptidase (nicastrin domain) was united with the catalytic function of an intramembrane aspartyl protease (presenilin active site). This enables the γ-secretase complex to execute the unusual events associated with RIP of type-I membrane proteins in the lipid bilayers. Here I and Dr. Gang Yu's lab list several ongoing experiments that were aimed at developing methods, protocols, and ideas that may further reveal details about the unique mechanisms the γ-secretase complex uses in executing its activity.

Ongoing Challenges, Approaches, & Results

The unusual nature of the γ-secretase complex evades classical enzymatic biochemistry. First it requires multiple protein components. Second it is endogenously tightly regulated. Finally it recognizes a growing number of substrates of a particular membrane class. This unusual enzyme cleaves amide bonds within the transmembrane regions of its substrates. Critical to its activity are the aspartates located in presenilin. The hydrolysis and the manner in which a growing list of substrates may be cleaved within a hydrophobic environment remains to be completey understood. Recently, (Kornilova, Bihel et al. 2005) proposed a location of an initial substrate-docking site that is distinct from the active site. It lies at the presenilin subunit interface. They used a photoaffinity probe designed as a potent helical peptide inhibitor that mimics the amyloid β-protein precursor substrate and binds to a site close to the active site. The location of the binding suggested that the immediate APP substrate passes between this site before it may access the acive site. However, whether this site is a true initial substrate-binding, docking-recognition site or an intermediary step remains to be determined. Furthermore, whether the γ-secretase complex may have evolved and adapted mechanisms for substrate specificity in recognition of a particular substrate over another remains a matter of debate and ongoing experimentation (Fraering, Ye et al. 2005). To address if the initial binding

site resides within the transmembrane regions of presenilin or if it may be part of nicastrins ability to initially recognize short alpha-amine stubs we generated an *in vitro* multi substrate competition assay (Fig. SF 1).

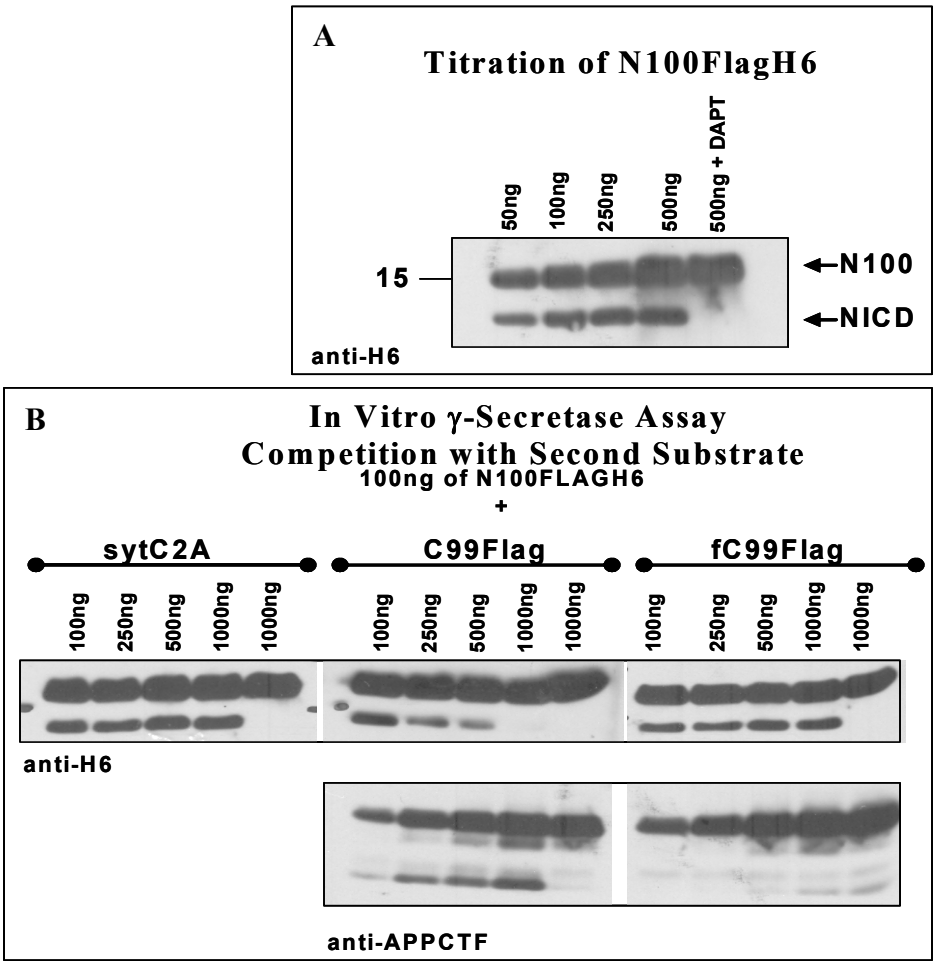


Figure SF 1. In vitro multi substrate competions assays. (A) Saturing amounts of NotchFLAG/H6, immediate γ-secretase substrate. As amount of substrate incubated with purified γ-secretase an increased in cleaved fragment is observed. (B) The reaction is carried out with multiple substrates. NotchFlag/H6 is added at a constant amount of 100ng. Titrated in the same reaction tubes are sytC2A, C99FLAG and fC99Flag. In vitro reactions are performed for 3hrs at 37◦C and then probed for the cleavage of Notch substrate (anti-H6) and C100 cleavage using (anti-APPCTF). Representive of four independent experiments.

The experimental results obtained, supports a model whereby the initial recognition of a substrate is determined by the recognition of the free alpha amine of the substrates. Generation of NICD increases in these invitro experiments as amount of starting substrate is increased. Similarly AICD increased as it is increasingly titrated into equal amounts of enzyme. Upon introducing a steady state level of N100 substrate and titrating sytC2A, C99FLAG, and fC99FLAG we find that NICD is only inhibited in samples where there is competing C99FLAG. This study supports a model where initial substrate recognition is soley dependent on the existence of a free alpha amine and not determined by the nature or identity of the substrate themselves. However, these experiments can not rule out steps inbetween substrate recognition and cleavage that may be further involved in substrate or cleavage specificity. Although a large number of substrates have been identified for the γ-secretase complex, studies regarding enzyme working mechanisms have predominantly revolved around the cleavage of Notch and C100 due to their obvious physiological and pathological significance.

The importance of the generation of free alpha amine of γ-secretase substrates through ectodomain shedding was previously established in assays primarily performed in a test tube. In an attempt to further establish the importance of the free alpha amine in

an intact cell based system as well as incorporate targeted specificity, we attempted to test if an intrabody approach may dirupt C99 recognition by the γ-secretase complex.

Intrabodies are engineered antibodies that allow for genes encoding antibodies to be manipulated so that the antigen binding domain can be expressed intracellularly. They have specific and high-affinity binding properties and are able to be stably expressed in precise intracellular locations inside mammalian cells. Our approach here was to transiently express single chain antibodies that were raised against the β-cleavage site of APP in cell lines set up for the C99GVP luciferase assay. An intrabody scFVC99 remains associated with APP during transport along the secretory line. It shields the β-secretase cleavage site. Additional intrabody scFVC99KDEL has an ER retention sequence that triggers APP disposal from the ER (Paganetti, Calanca et al. 2005). The luciferasae assay revealed significant inhibition of C99 cleavage by scFVC99KDEL, however, we observed an increase in fold activation of cells transiently transfected with scFVC99 (Fig. SF2).

Unfortunately in these experiments we are currently unable to differentiate if the fold activation caused by scFVC99 is due to an alternative, innocuous cleavage by α-secretase. A complimentary experiment may require testing the endogenous binding between Nicastrin and C99 in the presense of these intrabodies. This experiment assumes

that the current version of intrabodies have a high enough affinity to the short extracellular stub that in a cell based system would compete for recognition by nicastrin.

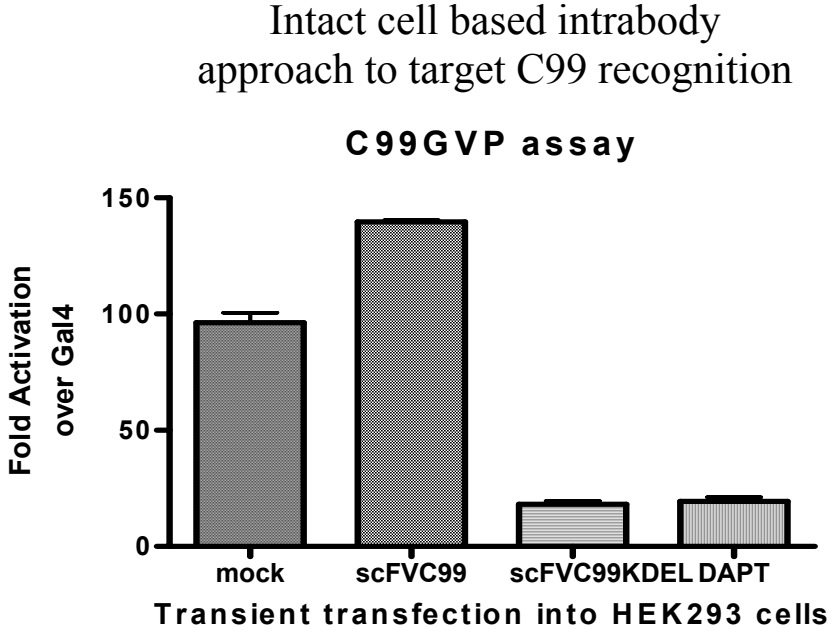


Figure SF 2. C99GVP luciferase assay with transiently transfected intrabodies. HEK293 cells were transiently transfected with all the constructions required to perform the transactivation assay. In addition, they were transiently transfected with single chain intrabodies scFVC99 as well as scFVC99KDEL or mock empty vector. As a control, parallel wells were treated with γ-secretase inhibitort DAPT. The transactivation assay is an indirect readout for γ-secretase activity. Although scFVC99KDEL is able to potently inhibit the cleavage of C99 we are unable to test if the mechanism of blocking the recognition of the short stub by these intrabodies is due to a competition with Nicastrin binding site. This particular experiment has been performed independently on more then four different occasions.

Regulated intramembrane proteolysis of Notch, APP, ErbB4, and the growing list of substrates cleaved by the γ-secretase complex require at least two proteolytic events: one between the extracellular and transmembrane domain, and second within the transmembrane domain. Approximately five years ago (Struhl and Adachi 2000), assayed for the substrate requirements for γ-secretase mediated (RIP) of Notch and other type I transmembarane proteins in vivo. They found that γ-secretase complex cleavage does not depend critically on the recognition of a particular sequence in these proteins but on the size of the extracellular domain. They found the smaller the size; the more efficiently they were cleaved. Thus, ectodomain shedding targets a class of membrane proteins for γ-secretase mediated cleavage. This thesis further builds upon the molecular mechanisms involved in recognition of such substrates. In addition, to the small size of the ectodomain stub and the free alpha amine we noticed that amino acid context (charge or small secondary structure) may contribute to how well a particular substrate is recongnized and thus cleaved within the transmembrane of a substrate. To experimentally test this further,

we took the approach of introducing small secondary structure (poly-proline helix (Fig. SF4)) or modified charge of the short (6 amino acid Asp residues) by site directed mutagenesis after a predicited signal peptide. These substrates were fused to the GAL4VP16 domains in an attempt to indirectly quatitate their fold transactivation (Fig. SF3).

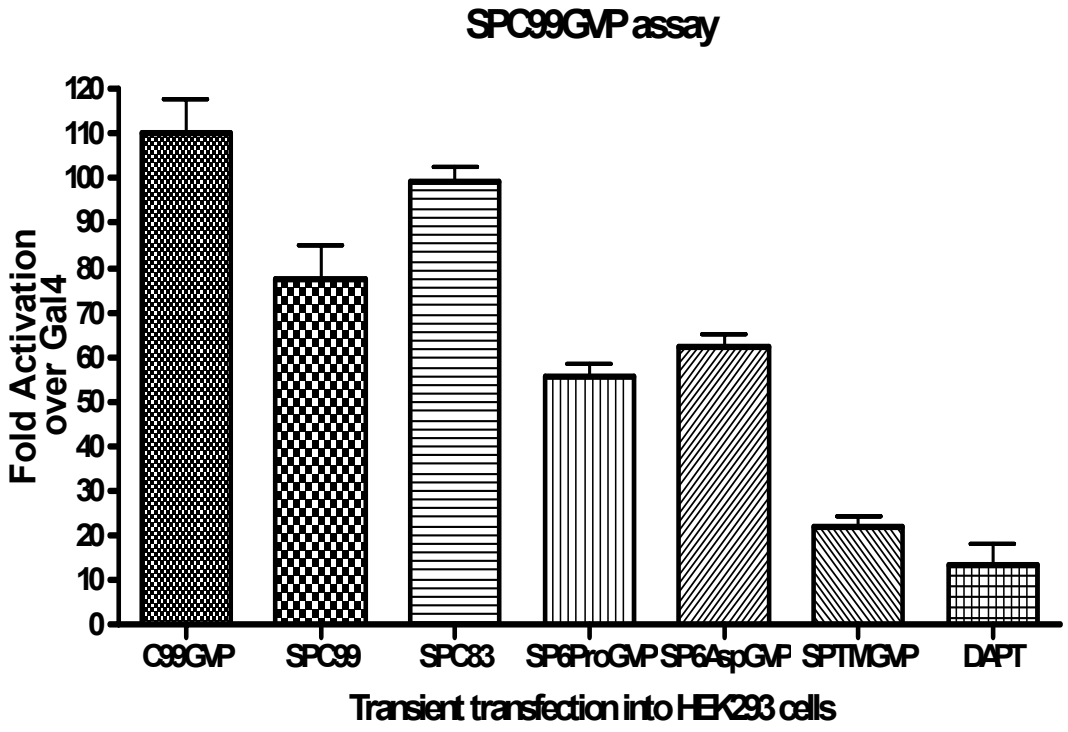


Figure SF3. Transactivation assay for C99GVP substrates that were modified in their N-terminal amino acid context. Substrates C99GVP were modified after predicted signal peptide cleavage either with 6 poly-proline sequences, or 6 charged aspartates or where the amino stub was completely removed. We find a slight decrease in fold activation of these modified substrates. Amino acid context around the initial recognition site of substrates may determine overall cleavage efficiency. How well these substrates are recognized by Nicastrin should be determined in future binding experiments.

We observed a modest decrease in the fold activation from substrates with (SP6ProGVP and SP6AspGVP). However, we can not rule out that a substantial portion of this activation may be due to the alternative α-secretase cleavage that would have removed the modifications introduced into these substrates. The SPTMGVP has all stubs removed but based on expression data may be a less stabley expressed protein and thus explain the significant reduction in transactivation. Future experiments will require designing substrates that may be differentiated between the fold activation observed from α-secretase cleaved substrate versus those seen from β-cleaved substrate.

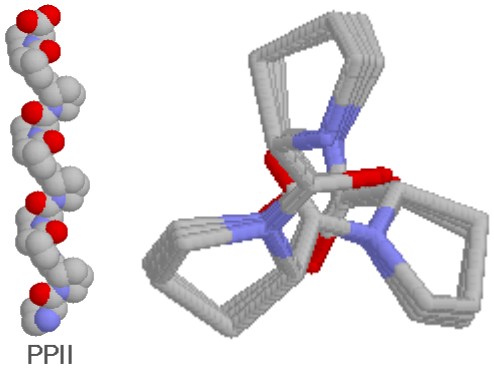


Figure SF4. Poly-Proline sequence and associated secondary structure from (PDB). A Poly-L-proline in PPII conformation, viewed parallel and vertical to the helix axis, presented as sticks, without H-atoms. PPII helix has a 3-fold symmetry, and every 4th residue is in the same position (at a distance of 9.3 Å from each other). This is important in when binding of PPII helices to SH3 domains.

A quantitative system amenable to revealing binding constants, sites of interaction, or serve as an assay for a small molecule screen may prove valuable in further understanding the working mechanisms of the γ-secretase complex. Towards this end, we decidided to test if we could incorporate a protein fragment complementation approach to more closely follow both *in vivo* and *in vitro* interactions between nicastrin

and its substrates. Protein fragment complementation assay (PCA) is an established strategy for determining protein-protein interactions based on protein interactions assisted folding of a rationally designed fragments of enzymes (Galarneau, Primeau et al. 2002). Assays may be developed for a quantitative analysis of small molecule induced protein interactions. In our assay design, we fused C-terminally a rapamycin-induced interaction of FKBP and yeast FRB (the FKBP-rapamycin binding domain of TOR (target of rapamycin)) to nicastrin and C99 substrates (Fig SF5). Generating mammalian cell based expression constructs for Nicastrin-FKBP-V5/H6, Nicastrin-FRB-V5/H6, C99-FKBP-V5/H6, and C99-FRB-V5/H6. These domains allow for control over homodimerization and heterodimerization events where two different engineered fusion proteins can be brought together by adding a small molecule dimerizer. Furthermore, the approach allows for bringing the events of nicastrin:substrate interaction under small molecule control. This particular project has advanced to the stage of completing the construct fusions. In the future, they may be checked for expression as well as be used in experiments to further quanitate binding constants, *in vivo* competition assays, and test if small molecule induced interactions may block additional substrate recognition by an active enzyme complex. Please refer to ARIAD Pharmaceuticals, Inc. ARGENT^{TM} regulated homodimerization and heterodimerization kits for more details regarding the fusion domains that have been incorporated in nicastrin and C99 substrates.

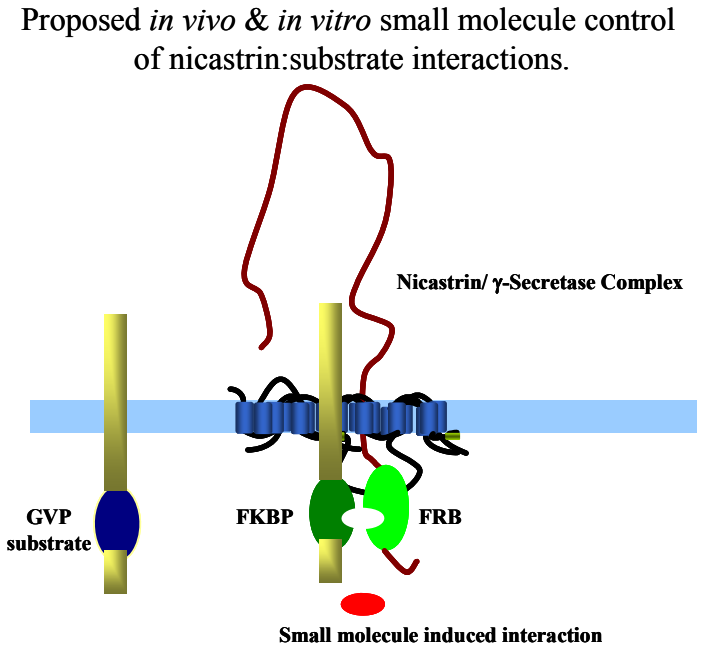


Figure SF5. An example of a small molecule blocking assay to further study mechanisms of nicastrin:substrate interactions in overall γ-secretase activity. γ-Secretase substrates and Nicastrin are fused with FKBP and FRB homo and hetero dimerization domains. The aim of these proposed experiments are to observe substrate recognition and cleavage under the control of small chemical molecules.

Additionally, ongoing experiments in the lab were aimed at elucidating the functional role of conserved γ-secretase components in a unicellular green alga, *Chlamydomaons reihardtii*, as well as the contributions they may make to the independent evolution of multicellularity in plants, such as *Arabidopsis thaliana*. Towards these projects, my contributions involved helping lab members organize a draft of a manuscript detailing aspects of γ-secretase function in green alga. In addition, I continue to contribute in effort of generating expression constructs of plant γ-secretase components. I am in the process of reconstituting this complex in our insect cell based system.

The future goals in Alzheimer's disease therapeutics remain developing approaches that will delay the onset (as has been accomplished in cardiovascular heart diseases) and slowing the progression (as for example the progress that has been made in Parkinson's disease). A continued understanding behind the working mechanisms of the γ-secretase complex is proving promising towards those must win goals.