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Review

Substrate specificity of γ-secretase and other intramembrane proteases

A. J. Beel and C. R. Sanders*

Department of Biochemistry and Center for Structural Biology, Vanderbilt University School of Medicine, Rm. 5142 MRBIII, 21st Ave. S., Nashville, Tennessee 37232-8725 (USA), Fax: +615 936 2211, e-mail: chuck.sanders@vanderbilt.edu

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Abstract. γ-Secretase is a promiscuous protease that cleaves bitopic membrane proteins within the lipid bilayer. Elucidating both the mechanistic basis of γ-secretase proteolysis and the precise factors regulating substrate identification is important because modulation of this biochemical degradative process can have important consequences in a physiological and pathophysiological context. Here, we briefly review such information for all major classes of intramembranously cleaving proteases (I-CLiPs), with an emphasis on γ-secretase, an I-CLiP closely linked to the etiology of Alzheimer's disease. A large body of emerging data allows us to survey the substrates of γ-secretase to ascertain the conformational features that predispose a peptide to cleavage by this enigmatic protease. Because substrate specificity *in vivo* is closely linked to the relative subcellular compartmentalization of γ-secretase and its substrates, we also survey the voluminous body of literature concerning the traffic of γ-secretase and its most prominent substrate, the amyloid precursor protein.

Keywords. Intramembrane, protease, proteolysis, secretase, rhomboid, substrate, S2P, amyloid precursor protein, I-CLiP, Alzheimer's disease, lipid raft, presenilin.

Introduction

Until recently a little known phenomenon, regulated intramembrane proteolysis (RIP) has emerged as a major pathway of signal transduction and cellular homeostasis [1, 2]. The remarkable feat of catalyzing a hydrolytic reaction within the hydrophobic interior of a lipid bilayer is carried out by a class of proteins known as intramembranously cleaving proteases (I-CLiPs). A variety of I-CLiPs have been discovered and are classified into three groups as outlined below [1]. Key properties of these unique enzymes are summarized in Figure 1.

Intramembranously-cleaving metalloproteases: site 2 protease family

Site 2 protease (S2P) is the founding member of the metalloprotease subfamily of I-CLiPs [3] and cleaves both the sterol regulatory element-binding proteins (SREBPs) and activating transcription factor 6 (ATF6) [2] in the Golgi membrane [4]. SREBP, the prototypical substrate of S2P, has two transmembrane (TM) domains connected by a short luminal loop with two large cytosolic N- and C-terminal domains [5]. SREBP is initially cleaved in its luminal domain by site 1 protease (S1P) and subsequently by an S2P-

* Corresponding author.

Protease Class	Signature Catalytic Motifs	Initial Cleavage by Another Protease Required for IP?	Helix Destabilization at Site of Cleavage Facilitates IP?	Substrate Trafficking Controls Access to Protease?	Existence of a Consensus Sequence at Substrate Cleavage Site?	Multiple Cleavage Sites per Substrate?
S2P	Metalloprotease	**HE**xx**H**, L**D**G	Yes	Yes	Yes	No	Unclear
Rhomboid	Serine protease	Gx**S**G, **H**	No	Yes	Yes	No	Yes
SPP	Aspartyl protease	Y**D**, LGxG**D**	Yes	Yes	Unclear	No	Yes
γ-secretase	Aspartyl protease	Y**D**, LGxG**D**	Yes	Possibly*	Yes	No	Yes

Figure 1. A summary of common themes among the I-CLiPs. Signature motifs are universally conserved among family members and are located in transmembrane regions of the protease. Residues in bold are directly involved in catalysis. With the exception of Rhomboid proteases, an initial ectodomain cleavage event facilitates subsequent intramembrane proteolysis (IP). The importance of helix destabilization is observed for virtually all I-CLiPs: flexibility of the transmembrane helix appears to be necessary for the protease to access the scissile region (*the potential importance of helix-destabilizing residues for γ-secretase-mediated IP is evaluated in this review). Trafficking of protease and substrate refers to whether these two entities are located in different organelles – a general control mechanism for IP that ensures that proteolysis occurs in a regulated fashion. In contrast to many other 'classical' proteases, the cleavage sites of I-CLiPs are generally not delineated by canonical sequences. Lastly, multiple sites of cleavage have been identified for many of these proteases.

mediated intramembranous cleavage, that releases the cytosolic N-terminal bHLH-Zip domain. This domain translocates to the nucleus and elevates transcription of genes involved in cholesterol biosynthesis [6]. Its liberation is strongly dependent on endogenous sterol levels [7] as a drop in the concentration thereof initiates changes in the subcellular localization of SREBP [8] (Fig. 2). The involvement of an escort protein, the SREBP cleavage-activating protein (SCAP), was detected during studies of cells that were resistant to modulation of proteolysis by sterol levels [9]. The modulatory properties of sterols derive from their ability to cause SCAP – and in turn SREBP (via an interaction between its C terminus and that of SCAP [10]) – to bind to endoplasmic reticulum (ER)-resident anchor proteins known as Insigs [11, 12]. In a similar manner, oxysterols bind to Insigs, thereby causing Insigs to bind to SCAP [13]. In either case, the anchoring of SREBP to Insigs prevents the translocation of SREBP from the endoplasmic reticulum to the Golgi, where it is proteolyzed. High sterol levels therefore regulate a negative feedback system: by inhibiting SREBP processing, the transcription of genes implicated in cholesterol biosynthesis remains at low levels. Sterol depletion abrogates the Insig-SCAP interaction, allowing the SREBP-SCAP complex to enter (COP)II-coated vesicles for anterograde transport to the Golgi [14], where SREBP is proteolyzed by S1P [15]. S2P proteolysis, which is not regulated by sterol levels, constitutively occurs after S1P proteolysis.

The precise factors disposing substrate to S2P proteolysis have been investigated, and primary structural characteristics within the TM domain (TMD) appear to be dispensable for the enzyme-substrate recognition process, akin to the current paradigm for substrate recognition by γ-secretase (discussed below) and some other I-CLiPs. Biochemical studies have identified sequence elements on both sides of the membrane that are required for efficient proteolysis, namely an arginine residue in the short luminal loop and a cytosolically located tetrapeptide sequence DRSR [16]. Substitution of the crucial arginine residue with alanine precluded proteolysis, whereas a more conservative substitution (lysine) did not have an inhibitory effect [16]. Later studies revealed that initial sterol-induced cleavage within the luminal loop by S1P, a membrane-embedded serine protease, occurs in proximity to the conserved arginine residue [5, 17]. Following S1P proteolysis, S2P cleaves SREBP substrates intramembranously between a leucine and cysteine [18], which is located three residues to the C-terminal side of the critical DRSR motif. However, neither the leucine nor cysteine was found to be necessary for cleavage, nor were many of the conserved residues within the transmembrane domain, as shown by singly replacing each TM residue with alanine [18]. These data suggest that strict conservation of the sequences proximal to the cleavage site and within the TMD is unnecessary for S2P activity. However, at least one residue of the universally conserved asparagine-proline (NP) pair is required. This dipeptide, which is located in the middle of the first SREBP TMD, has been proposed to facilitate unwinding of the transmembrane α-helix, thereby potentiating exposure of backbone residues in the protease active site [19].

Five features observed for the cleavage of SREBP – the requirement for interruption of the transmembrane helix, the dispensability of primary structure

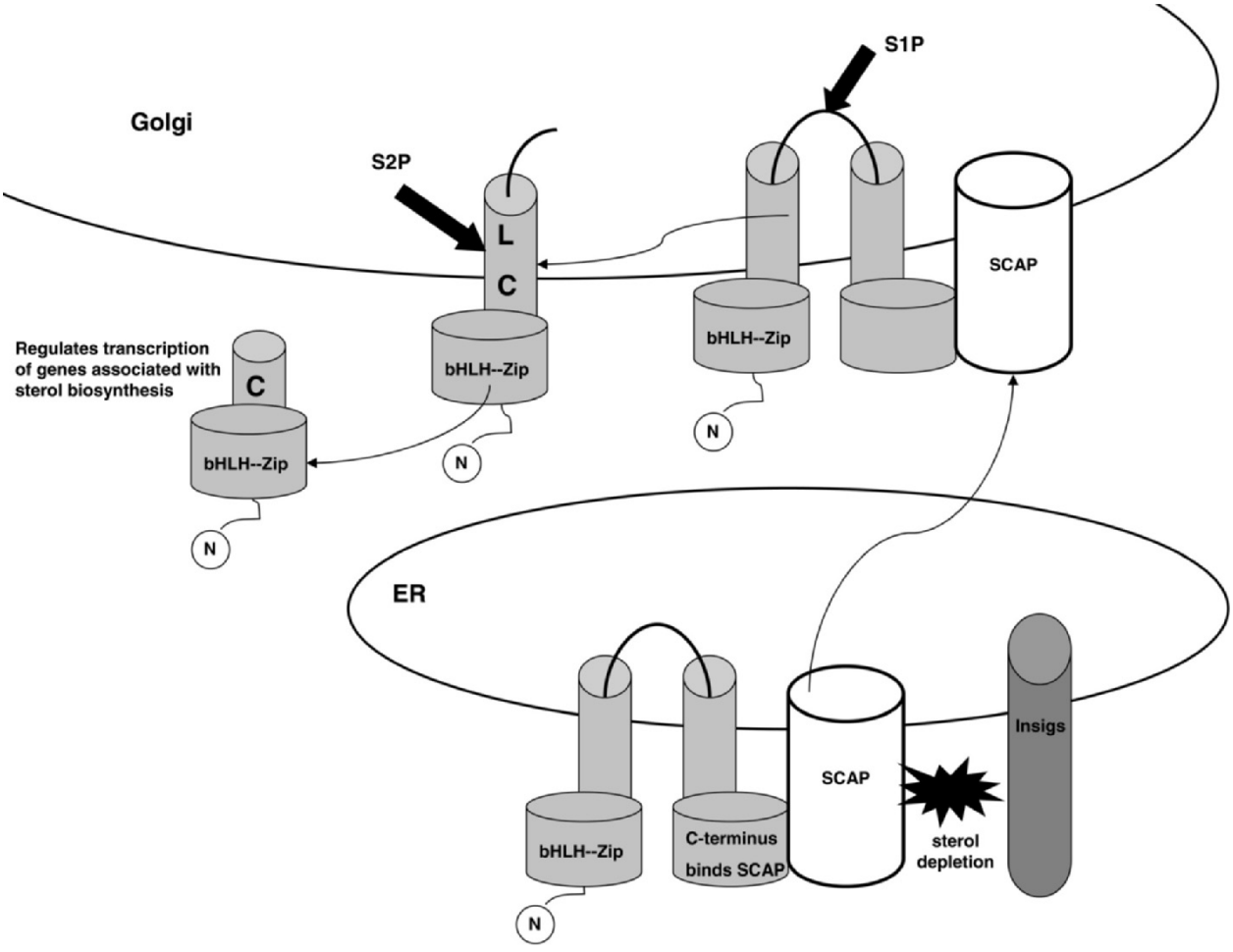

Figure 2. Proteolytic pathway of SREBP. SREBP initially coexists in the endoplasmic (ER) with its escort protein, SCAP (bottom panel). The presence of sterols or oxysterols promotes an interaction with ER-resident anchor proteins known as Insigs. Sterol depletion abrogates this interaction and the SREBP-SCAP complex translocates to the Golgi, whereupon SREBP is initially proteolyzed in its short luminal loop by S1P, a membrane-embedded serine protease. Following this cleavage, the transmembrane region of the SREBP N-terminal fragment is proteolyzed intramembranously by site 2 protease (S2P) between a leucine and a cysteine, which results in liberation of the N-terminal domain. This domain is a transcription factor that translocates to the nucleus and up-regulates expression of genes involved in cholesterol biosynthesis. SCAP and Insigs are polytopic membrane proteins.

immediately proximal to the cleavage site, a preliminary ectodomain cleavage as a prerequisite for intramembranous proteolysis, the involvement of escort proteins, and the release of biologically active factors – are not unique to S2P but reflect features for other enzymes of the I-CLiP family.

Intramembranously cleaving serine proteases: rhomboid family

In *Drosophila* the founding member of the rhomboid serine protease family – Rhomboid-1 – is responsible for the proteolysis of Spitz [20], the liberated domain of which is involved in signaling through receptor tyrosine kinases [21]. Rhomboids are ubiquitously present in all forms of life on earth, and participate in diverse biological pathways such as quorum-sensing in *Providencia stuartii* and signaling via the epidermal growth factor (EGF) receptor in *Drosophila.* Rhomboid is also implicated in a number of human diseases, such as type II diabetes and autosomal-dominant optical atrophy [20]. Rhomboids were initially believed to employ a catalytic triad (Asx, His, Ser) akin to classical serine proteases such as trypsin, chymotrypsin, and elastase; however, recent studies have indicated that the Asn residue of rhomboid is dispensable for catalytic activity, leading to a catalytic dyad model of proteolysis [22], which has been corroborated by crystallographic analyses of rhomboid proteases [23–25]. These studies revealed a catalytic dyad buried within the membrane, in addition to novel structural features that permit substrate selectivity and intramembrane proteolysis. GlpG, a rhomboid from *Escherichia Coli*, possesses six TM helices (TM1–6). As a consequence of helix-destabilizing glycine residues, TM4 is shorter than a

typical membrane-spanning alpha-helix. This results in an aqueous cavity in the membrane plane, surrounded on three sides by TM helices and solvent-exposed on the fourth to the depth at which the N-terminal end of TM4 originates [23, 24]. It is within this hydrophilic cleft that scission of a TM substrate is believed to occur. Ben-Shem et al. [24] suggested that substrate helix destabilization permits contact between the enzymatic active site and the scissile bond at a crevice between TM2 and TM5. TM5 is believed to act as a dynamic gating system for substrate entry to the active site [26]. The problem of bringing water into the lipophilic context of the bilayer is resolved by the crystallographic data – the curtailed length of TM4 and the provision of polar residues by other TM helices surrounding the active site permit water accessibility within the plane of the bilayer [27]. Wang et al. [23] propose that the upper segment of the TM helix of the substrate unfolds within the active site, thereby exposing the scissile bond to hydrolysis. The crystal structures of GlpG provide information about its mechanism of intramembrane proteolysis; however, the lack of an apparent evolutionary relationship between rhomboid-type proteases and other I-CLiPs precludes generalization of this mechanistic information [28].

Rhomboid proteolysis, like that of S2P, also depends on the relative subcellular localization of the various components involved in the process. Indeed, the similarities between this pathway and that of SREBP proteolysis are striking. Spitz, a prototypical substrate for rhomboid proteases in *Drosophila*, is constitutively localized to the ER, whereas Rhomboid is found in the Golgi apparatus [21]. Cleavage of Spitz is therefore regulated by its intracellular traffic, which requires involvement of an escort protein, Star. Cotranslocation of Star and Spitz from the ER to Golgi allows interaction between Rhomboid and Spitz, leading to proteolytic degradation of the latter and its subsequent export from the cell [21, 29].

Studies of the sequence requirements for Rhomboid substrates have demonstrated an importance for helix destabilization, analogous to that observed for S2P substrates. Insertion of the Spitz TMD into other proteins that are normally not cleaved by rhomboid proteases led to the degradation of the chimeric constructs, suggesting that the essential features for proteolysis are contained within the TMD of Rhomboid substrates. Later analyses revealed that a conserved motif in the Spitz TMD with low helical propensity provided the basis for Rhomboid substrate specificity [30]. Thus, substrate conformation and/or dynamics determine its susceptibility to Rhomboid cleavage, as was observed for S2P. Urban and Freeman [30] found that the luminal portion of the TMD was most critical for Rhomboid proteolysis. Two consecutive residues (GA) contained therein, when inserted into non-substrates, allowed these peptides to be processed efficiently. Insertion of β-branched amino acids, which decrease local helical propensity, also enhanced proteolysis [30]. Another recent study confirmed the importance of helix-destabilizing residues; however, no consensus sequence was identified, and different members of the Rhomboid family were shown to exhibit distinct substrate preferences [31], suggesting that substrate recognition involves other structural elements distal from the cleavage site. One aspect of Rhomboid proteolysis that differs significantly from processing by other I-CLiPs is that Rhomboid does not require preliminary ectodomain shedding, and is able to cleave full-length substrates with large extracellular domains [22, 30].

Intramembranously cleaving aspartyl proteases: presenilin, signal peptide peptidase, and others

Presenilin (PS) and signal peptide peptidase (SPP) are members of an I-CLiP subfamily whose catalytic mechanism involves two aspartate residues, akin to classic aspartyl proteases [32]. Both are polytopic membrane proteins with two putative aspartate residues demarcating the catalytic site [33–35]. The observation that transition state analogues can inhibit both enzymatic activities suggests that the two share similar active sites [36, 37]. Though similarities between PS and SPP abound, differences have also been documented. Whereas PS is a member of a multiprotein complex, SPP can act alone [38]. The two proteases exhibit different substrate recognition profiles: PS cleaves type I single-TM proteins (cytosolically oriented C terminus), whereas SPP cleaves inversely oriented type II TM proteins [32, 39]. SPP and PS have conserved catalytic motifs (YD and LGxGD), each containing one of the active site aspartates, located in adjacent TM regions. Intriguingly, the orientation of these two motifs is inverted in each of the two proteases, a property that may account for their unique *in vivo* substrate preferences [40] (Fig. 3). SPP cleaves certain signal peptides with type II transmembrane orientation following their liberation from a preprotein by signal peptidase (SP) [41, 42]. In addition to its role in the degradation of remnant signal peptides, SPP generates biologically active peptides, such as HLA-E epitopes and calmodulin-dependent signaling peptides [43]. Furthermore, given its role in processing the hepatitis C virus (HCV) core protein [44] and the GB virus B core protein [45], SPP may be a useful therapeutic target in the prevention of certain viral infections. In analogy to S2P and Rhomboid, SPP requires helix-breaking residues for proteolysis [41], while its substrates appear to lack a well-defined recognition sequence [46].

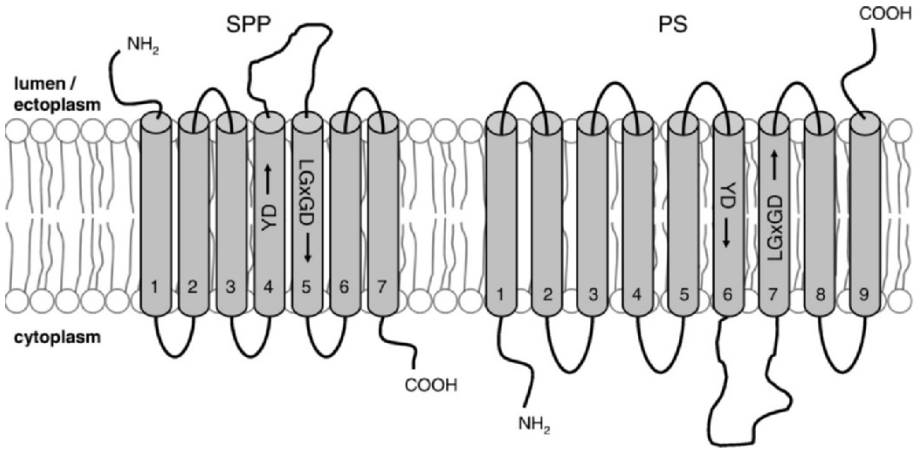

Figure 3. Topologies of signal peptide peptidase (SPP, left) and presenilin (PS, right). Note the inverted orientations of their respective YD and GxGD catalytic motifs, a property that likely accounts for the difference in the preference of each enzyme for substrate orientation. This figure has some similarity to a figure presented previously [33].

Low-resolution structural data of γ-secretase allow us to speculate on the mechanism it employs to accomplish hydrolytic cleavage within the bilayer. An electron microscopic single-particle reconstruction of the γ-secretase complex supports the notion of an intramembrane aqueous cavity through which water is able to penetrate the bilayer [47]. Li et al. [60] suggest that a number of TM helices of PS provide a protein-surrounded cavity within the bilayer that is amenable to the presence of water. Studies employing cysteine-scanning mutagenesis and measurement of the accessibility of artificial cysteine residues to a hydrophilic reagent have corroborated the notion of an aqueous cavity within the interior of γ-secretase [48, 49].

Profile of an enigmatic protease: γ-secretase

γ-Secretase is perhaps the most intensely studied intramembrane protease because of its critical role in the neuropathogenesis of Alzheimer's disease (AD) [50, 51]. It processes the C-terminal fragment (CTF)-derived portion (C83 or C99) of the amyloid precursor protein (APP) within the membrane, liberating the beta-amyloid peptide (Aβ), which has a proclivity for aggregation, forming amyloid plaques in the central nervous system. Intermediates in the formation of these neuritic plaques are thought to be a major factor in the etiology of AD [52]. Despite its pivotal role in the most economically costly disease in the developed world [53, 54], many basic features of the γ-secretase complex remain to be elucidated.

Composition, assembly, and subcellular localization of γ-secretase

Unlike other members of the family, γ-secretase requires multiple components – PS, nicastrin (Nct), anterior pharynx defective-1 (Aph-1), and presenilin enhancer-2 (Pen-2) – for enzymatic activity both *in vivo* [55, 56] and *in vitro* [57]. PS, a nine-pass TM protein [58, 59], is the catalytic core of the protease [35, 60, 61]; Nct, a single span membrane protein with a large, heavily glycosylated extracellular domain, assists with substrate selection [62]; Aph-1, a seven-pass TM protein, promotes the assembly, stabilization, and traffic of the complex [63]; and Pen-2, a two-pass TM protein, triggers PS endoproteolysis [64–66]. The precise molecular stoichiometry of the complex was recently determined by Sato et al. [67] to be 1:1:1:1 for PS:Nct:Aph-1:Pen-2. The relevance of non-essential cofactors remains uncertain.

Proper complex assembly and maturation requires all four components and appears to occur early in the secretory pathway [68, 69], though the process has yet to be precisely localized. Assembly begins with the formation of a subcomplex between Nct and Aph-1, which is mediated by interactions between their respective TMDs [70]. Full-length presenilin stably associates with the initial Nct-Aph-1 complex [71, 72] via an interaction between the extreme C-terminus of PS and the TMD of Nct [73–75]. Interaction with Pen-2 via TMD4 of PS [76] then enables full-length presenilin to execute auto-endoproteolysis to form NTF and CTF heterodimers, which constitute the active protease [72, 64–66]. The PS-NTF/CTF heterodimer is stabilized via an interaction with the C-terminal domain of Pen-2 [77].

The subcellular localization of γ-secretase has been a controversial issue, as residence has been reported in numerous compartments, including the ER [68], the Golgi/trans-Golgi network (TGN) [78], the endosome [79], the lysosome [80], the phagosome [81], the autophagic vacuole [82, 83], and the plasma membrane [84, 85]. Investigation using quantitative immunogold-electron microscopy reported PS1 localization at the plasma membrane, the ER, endosomes, vesicular-tubular clusters (VTCs), the nuclear envelope, and COPI-coated vesicles, while PS1

localization at the Golgi was minimal [86]. Despite the substantial concentration of PS in the ER, studies have demonstrated that γ-secretase attains an active proteolytic state in post-ER compartments [87]. The precise contribution of each of these locales to overall cellular γ-secretase activity has not been evaluated, though evidence suggests the plasma membrane and endosomes to be the principal sites of proteolysis [88].

Heterogeneity of γ-secretase

PS and Aph-1 exist in multiple isoforms, each of which can be selectively incorporated into γ-secretase to generate an active protease, suggesting the possibility of distinct γ-secretase activities.

Two isoforms of PS – PS1 (the major isoform) and PS2 – exhibit a number of notable differences [89]: (1) there are strong phenotypic differences between PS1– and PS2– systems; (2) isoform-specific expression levels are variable among different tissues; (3) each is incorporated into a proteolytic complex in a mutually exclusive manner; (4) each isoform exhibits differential susceptibility to certain γ-secretase inhibitors; (5) the two isoforms differ in their ability to process APP CTFs. Furthermore, there is evidence for tissue-specific alternative splicing of the presenilin transcripts [90].

Three isoforms of Aph-1 have been identified in humans: Aph-1a (for which there are two splicing variants – Aph-1aS and Aph-1aL) and Aph1-b [91]. As with PS, tissue-specific expression patterns of Aph-1 have been documented [92], and evidence suggests these isoforms may be associated with functionally distinct γ-secretase complexes [93]. Coimmunoprecipitation experiments implicated Aph-1aS, Aph-1aL, and Aph-1b in distinct γ-secretase complexes, each of which contained either PS1 or PS2, demonstrating the existence of six distinct proteolytic entities in mammals [94]. The functionality of each of the six combinatorial possibilities has recently been confirmed [95].

The factors regulating expression levels of the various isoforms and the biological significance of each of the six constitutionally distinct complexes are not known. A greater understanding is necessary to determine whether functionally distinct γ-secretase complexes exist at different stages of development or in different cell types, and if the variant complexes exhibit different substrate recognition properties or different intracellular trafficking.

Ectodomain shedding of γ-secretase substrates

The substrates for γ-secretase are generally derived from large precursor proteins that undergo a prerequisite ectodomain shedding event before being cleaved by γ-secretase [96]. PS-dependent proteolysis therefore culminates a sequential proteolytic cascade that begins with the removal of a large extracellular fragment. This antecedent cleavage occurs in proximity to the membrane, such that the residual ectodomain is typically shorter than about 30 residues, a property that dramatically enhances substrate recognition by γ-secretase [96]. The enzymes that catalyze the ectodomain shedding event are termed sheddase*s* and include members of the disintegrin and metalloprotease (ADAM) family, such as ADAM-10 and ADAM-17; aspartyl proteases such as the β-site APP-cleaving enzymes (BACE1), and matrix metalloproteases (MMPs) [97]. ADAM-17, otherwise known as tumor necrosis factor-α (TNFα)-converting enzyme (TACE) and ADAM-10 (and its ortholog Kuzbanian) are thought to exist predominantly at the cell surface, though intracellular shedding by these two proteases has also been documented [reviewed in ref. 98]. Together with ADAM-9, these metalloproteases constitute 'α-secretase' activity, which processes APP in a non-amyloidogenic fashion [99]. BACE1 (the putative β-secretase that processes APP in the amyloidogenic pathway) is a TM aspartic protease with maximal activity within the lumen of acidic organelles such as endosomes and also the Golgi apparatus [reviewed in ref. 100).

The ectodomain shedding event that generates PS substrates is often controlled by changes in higher-order structure, such as ligand-induced oligomerization (Fig. 4). The binding of ligand or the *cis* homo- or heterodimerization of a receptor with another receptor (e.g., homophilic association of cadherins) has been observed to modulate ectodomain shedding for a large number of PS substrates. For example, the binding of CD44 to hyaluronan oligosaccharides [191] or to a monoclonal antibody [192] can promote ectodomain shedding. The EphB2/ephrinB2 system involves reciprocal regulation in which both EphB2 (receptor) and ephrinB2 (ligand) are metalloproteolyzed upon *trans* interaction between the two proteins located on adjacent cell surfaces [136, 137]. Furthermore, changes in substrate glycosylation state, which may affect ligand binding, are known to affect ectodomain shedding [168]. Preshedding dimerization or oligomerization has been reported for a number of γ-secretase substrates, including APP [193, 194], APLP-1 and APLP-2 [194], syndecan-3 [195, 196], and the homophilically associating cell adhesion molecules (CAMs), including the cadherins [197] and protocadherins [198], nectin [199], and L1 [200].

Substrate docking by γ-secretase

Nct, a single-span TM protein component of PS, was initially reported to be critical for the maturation of γ-

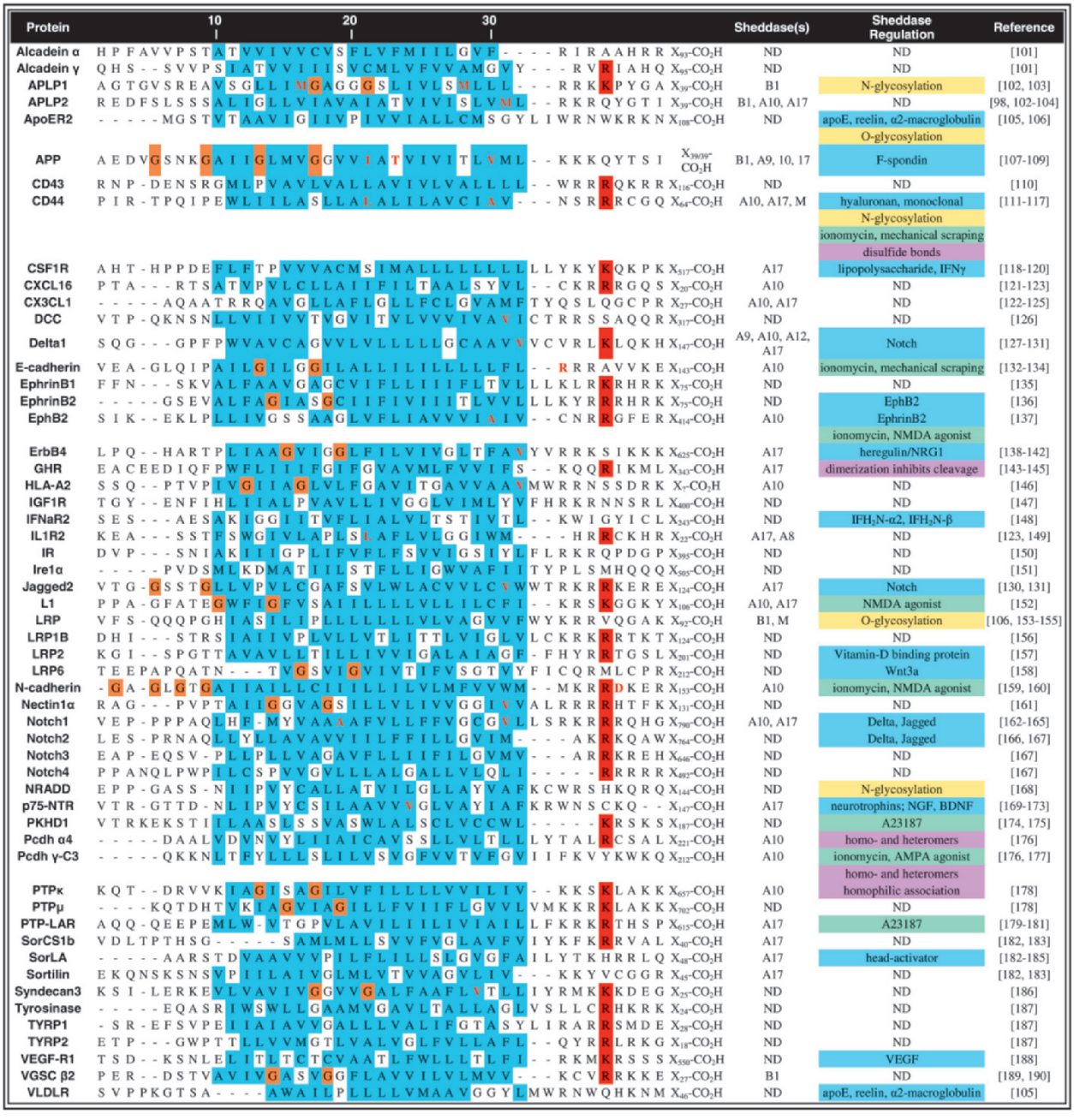

Figure 4. Sequences and properties of γ-secretase substrates. The sequences of 55 known type I membrane protein substrates are presented. Only the first 8 residues of the extracellular domain (ECD; unless actual length is shorter) and the first 8 residues of intracellular domain (ICD) are given. The blue region of the multiple sequence alignment (MSA) corresponds to hydrophobic TM residues; the column of arginine and lysine residues highlighted in red are part of stop-transfer signals; the orange-shaded glycine residues are parts of GxxxG motifs (which may be dimerization-prone). γ-Secretase cleavage sites, where known, are immediately to the left of residues displayed in red. Appropriate references for PS-mediated proteolysis and ectodomain shedding are provided. The colored boxes in the column entitled "Sheddase Regulation" correspond to the known regulatory modes for prior ectodomain shedding of a particular γ-secretase substrate. Blue boxes indicate that ligand binding controls cleavage, with the cell entries containing the names of the responsible ligand(s). Yellow boxes indicate that differential glycosylation influences ectodomain cleavage. Green boxes indicate that calcium influx promotes ectodomain cleavage: increases in cellular calcium concentrations, either by introduction of an ionophore, mechanical scraping of cells, or use of an NDMA or AMPA receptor agonist, likely directly up-regulate metalloprotease activity. Red boxes indicate that substrate oligomerization affects cleavage. These various regulatory modes are not mutually exclusive and for cases in which multiple modes affect the cleavage of a substrate, the individual contribution of each stimulus has generally not been evaluated. A10, ADAM10/kuzbanian; A17, ADAM17/TACE; B1, BACE1; M, MT1-MMP. The multiple sequence alignment was performed using the bcl_align program (Jens Meiler, unpublished data) and used data from various protein structure prediction algorithms. The chromatic visualization was done using Jalview.

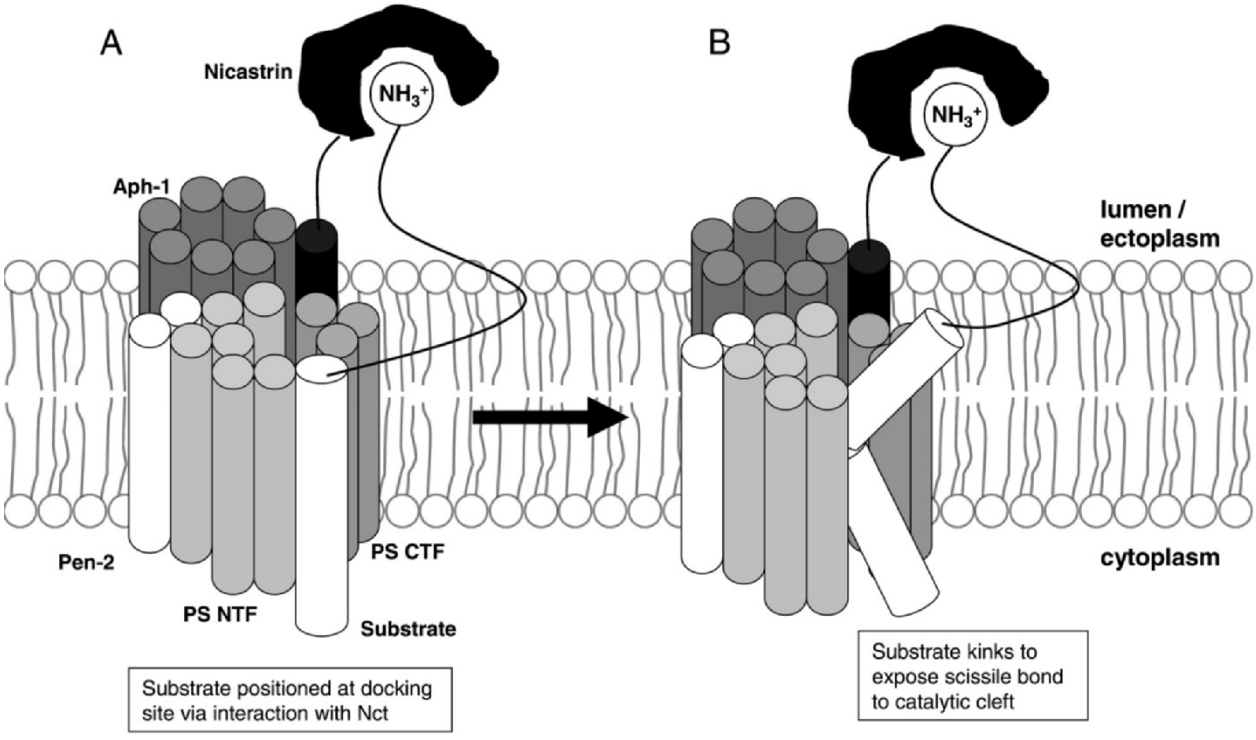

Figure 5. Model for γ-secretase substrate recognition and cleavage. (*A*) Following ectodomain shedding, the truncated extracellular amino terminus of the substrate (white) makes electrostatic contact with E333 of the nicastrin ectodomain. This interaction positions the substrate at the initial substrate-binding site of the complex, located at the interface between the two fragments of the PS heterodimer. (*B*) The substrate then either physically translocates or kinks (shown) in such a way that the scissile bond is positioned within the catalytic site formed by the transmembrane aspartate residues of PS helices 6 and 7. This model reflects aspects of figures from Wolfe [51], Shah et al. [62] and Kornilova et al. [207].

secretase and for protease-substrate binding [201]. Later studies by Shah et al. [62] cogently demonstrated that Nct assists the substrate recognition process. Following preliminary ectodomain shedding of a γ-secretase substrate, the large Nct ectodomain binds to the substrate's free amino terminus. Chemical or antibody-mediated obstruction of substrate N-terminal amino functionality drastically inhibits substrate binding and γ-secretase activity. Shah et al. [62] identified a region within the ectodomain of Nct – the DAP domain (DIYGS *a*nd *p*eptidase homologous region) – to be critical for substrate recognition. Various mutations of a highly conserved glutamate residue in the DAP domain abrogated substrate recognition, whereas mutation of this residue to an aspartate did not significantly interfere with PS activity. These results suggest that the carboxylate side chain of Nct E333 is involved in a direct interaction with the free N-terminal amino group of γ-secretase substrates (Fig. 5) [62].

The interaction between Nct and the substrate N-terminus is involved in positioning the substrate into the initial substrate-binding site (or docking site), which is spatially distinct from the catalytic site. Multiple lines of evidence support the existence of physically separated docking and catalytic sites, including (1) the ability of γ-secretase to bind simultaneously a substrate and a transition state analog (TSA) inhibitor [202, 203]; (2) the observation that TSAs exhibit non-competitive inhibition [204, 205]; (3) FRET analysis demonstrating proximity between APP CTFs and PS while PS is bound to a TSA inhibitor [206].

An elegant study by Kornilova et al. [207] demonstrated that the putative docking site and catalytic site are separated by a distance not exceeding three amino acid residues in length. A 10-residue helical peptide γ-secretase inhibitor (D-10) was reported to bind to the PS heterodimer interface. In contrast to TSA inhibitors, which allow simultaneous binding by substrates, D-10 prevented substrate binding, suggesting that it occupies the docking site. Whereas D-10 (at the docking site) and TSA inhibitors (at the active site) have been shown to simultaneously bind γ-secretase, D-13 (generated by a three-residue extension of D-10) prevented binding of TSA inhibitors, suggesting that D-13 occupies both the docking site and the active site [207].

Such studies have led to a model of catalysis in which an interaction between Nct and the substrate N terminus positions the substrate within the docking

site, at the interface between PS NTF and CTF. Either physical translocation of the entire substrate or a kink within the TM region of the substrate enables the entire substrate or a portion thereof, respectively, to enter the catalytic site where peptide scission occurs (Fig. 5). Position x of the LGxGD catalytic motif located in TM7 of PS has been shown to be critical for substrate recognition [208].

The role of lipid rafts in γ-secretase action

A large body of evidence has implicated cholesterol in the production of Aβ and the progression of AD. Cholesterol depletion in hippocampal neurons was shown to entirely abrogate Aβ production [209], while hypercholesterolemic mice have been shown to be afflicted with an accelerated amyloid pathology [210]. Cholesterol reduction (via application of the cholesterol-lowering agents known as statins) was shown to reduce BACE processing of APP in HEK 293 cells [211] and to reduce Aβ generation both in hippocampal neurons and in guinea pigs [212]. Epidemiological studies have correlated a reduction in the prevalence of dementia with the use of statins [213, 214]. These effects may be related to disruption of lipid raft structures, in which BACE and γ-secretase have been reported to reside [215–218]. Indeed, emerging evidence suggests an intimate connection between APP processing and lipid rafts [summarized in ref. 219]. Note, however, the existence of contradictory epidemiological data [220], and the ambiguity characterizing the relationship between statins and Aβ, which could, for instance, be a consequence of altered protein isoprenylation [221].

Lipid rafts are highly ordered membrane microdomains characterized by elevated levels of cholesterol, sphingolipids, glycolipids, saturated phospholipids, and glycosylphosphatidylinositol (GPI)-anchored proteins relative to the bulk environment of predominantly freely mixing unsaturated phospholipids [reviewed in ref. 222]. This particular arrangement imparts the property of resistance to solubilization by non-ionic detergents, such as Triton X-100, at reduced temperature, and delineates lipid rafts (also called detergent-resistant membranes, or DRMs) as a unique subphase within the context of the lipid bilayer. The functional importance of lipid rafts derives from their ability to sequester various proteins critical to such biological processes as signal transduction, membrane traffic, lateral membrane sorting [222], and, perhaps, regulated intramembrane proteolysis [reviewed in refs. 223, 224].

Various reports have corroborated an intimate relationship between rafts and APP processing. APP has been reported to interact with resident raft proteins [225], and studies have demonstrated APP processing to be affected by ceramide, sphingolipids, glycosphingolipids, and isoprenoids [226–230]. The critical dependence of amyloidogenic processing on lipid rafts [231, 232] led Ehehalt et al. [231] to postulate the existence of two separate pools of APP – a raft-associated one that is cleaved by BACE in an endocytosis-dependent manner, and a contingent external to lipid rafts capable of undergoing non-amyloidogenic processing by α-secretase, most likely at the cell surface. Indeed, reduction of cholesterol levels has been shown to enhance non-amyloidogenic (α-secretase-mediated) processing of APP, an effect that correlates with augmented membrane fluidity and impaired APP internalization [233]. Vetrivel et al. [234] have provided experimental support for a model in which γ-secretase and its substrates are compartmentalized into discrete membrane microdomains. Distinct microdomain locales have also been reported for a γ-secretase substrate and its sheddase [235].

While it remains to be seen whether cleavage by other γ-secretase substrates is promoted by association with raft domains, it is very likely that mechanisms governing the movement of substrates between membrane microdomains are important. Several lines of evidence suggest that ligand binding and oligomerization may control association of membrane proteins with lipid rafts. For example, translocation of the B cell antigen receptor to lipid rafts is triggered by antigen-induced oligomerization, following which signal transduction events occur [236]. Similarly, the T cell antigen receptor [237] and FcεRI [238] are recruited to rafts immediately subsequent to ligand binding. Engagement of tumor necrosis factor (TNF) receptor 1 by TNFα also triggers its association with rafts [239]. Binding of γ-secretase substrates, many of which function as TM receptors, with cognate ligands may also trigger association with rafts. As discussed earlier, the ectodomain shedding event for a large number of γ-secretase substrates is triggered by ligand-binding or oligomerization, which suggests that ectodomain shedding is linked to the promotion of the affinity of certain substrates for rafts. Indeed, studies have reported the association of a number of γ-secretase substrates with lipid rafts and closely related caveolae [240], including ApoER2 [241], CD44 [242], DCC [243], IGF-1R [244], LRP-1 [245], and N-cadherin [246]. Moreover, ligand-triggered association with rafts has been demonstrated for EphrinB1 [247], EphB2 [137], ErbB4 [248], GHR [249], HLA-A2 [250], and LRP-6 [251], while phosphorylation-dependent recruitment has been demonstrated for p75 [252].

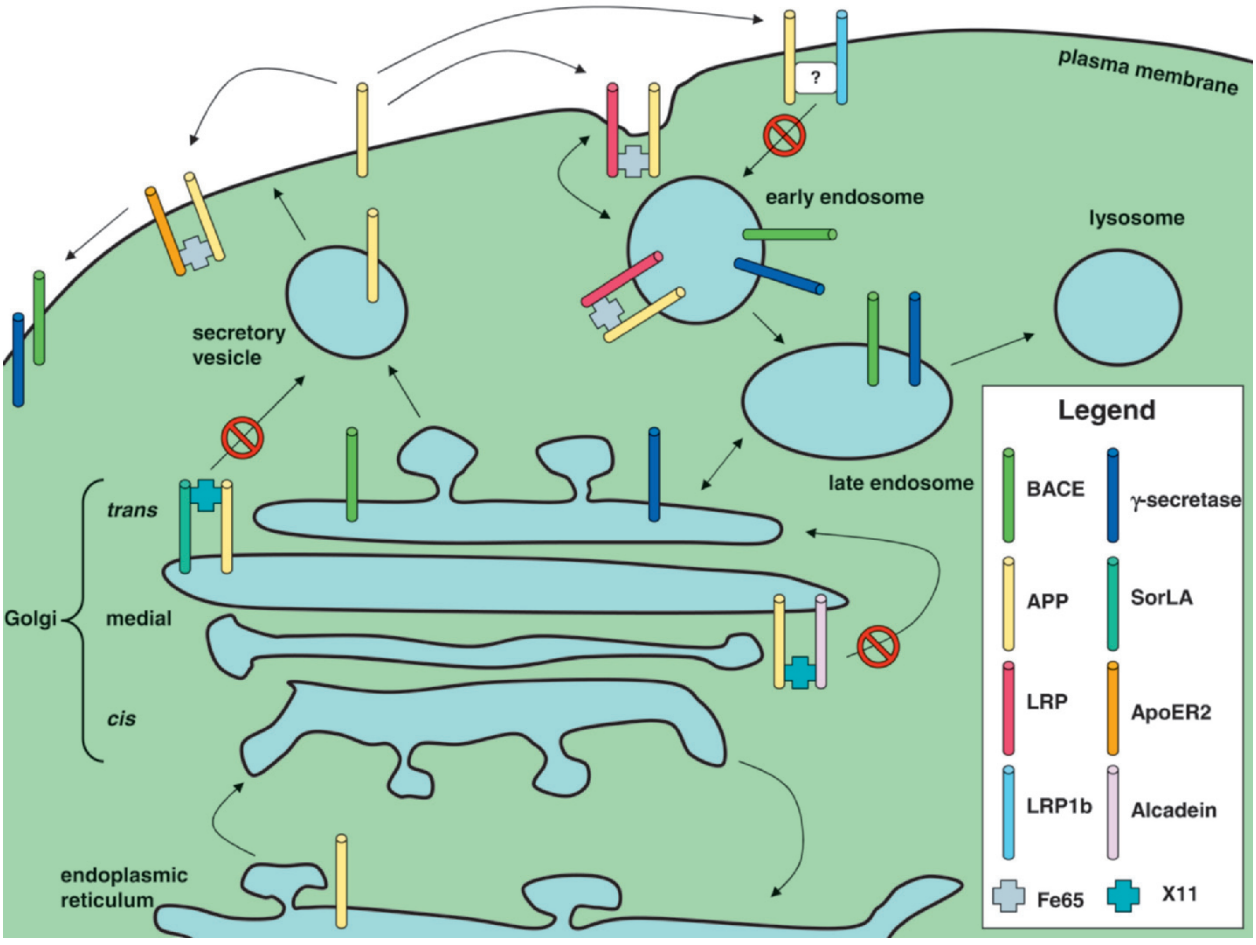

Figure 6. Regulating access of APP to γ-secretase by subcellular trafficking. APP (yellow cylinder) undergoes complex maturation and trafficking throughout the endosomal system and may be cleaved by secretases in a number of subcellular compartments. The majority of amyloidogenic (BACE) processing appears to occur after internalization of cell surface APP, possibly in conjunction with LRP (red cylinder) and the adaptor protein, Fe65 (gray cruciform). Myriad proteins have been reported to bind to and modulate the trafficking and processing of APP. Many of these interactions may be mediated by cytosolic adaptor proteins (denoted by the question mark signs and the cruciforms). Much of the information presented in this figure must be subjected to additional experimental scrutiny. This figure also does not include the additional level of trafficking that occurs between raft and non-raft fractions of the membrane, nor does it display BACE or γ-secretase trafficking. The activity of these two proteases has been demonstrated to occur at the cell surface and in various compartments of the endosomal system, with the latter thought to represent the principal site of activity.

Controlled intracellular trafficking as a proteolytic regulatory mechanism – the example of APP

APP traffic has been extensively studied; however, many aspects remain ambiguous and controversial. Traffic-controlled access of substrate to protease is dynamically regulated for several I-CLiPs (cf. Fig. 1), and will be surveyed in this section for the specific case of γ-secretase and APP (Fig. 6).

Myriad proteins have been identified as mediators of APP traffic, many of which are members of the low density lipoprotein (LDL) receptor and phosphotyrosine-binding (PTB) domain families. APP processing occurs at a number of locations as it is trafficked throughout the cell: prior to reaching the cell surface, at the cell surface, and following internalization from the cell surface, although the precise contribution of each pathway has not been investigated.

LDL receptor-related protein (LRP) has been implicated in the raft association, internalization and amyloidogenic processing of APP [253–255]. LRP physically associates with uncleaved APP [256] and BACE1 [257], and may therefore be involved not only in the endocytosis of APP but in its presentation to BACE1. LRP1b, which has a reduced rate of internalization relative to LRP, impairs APP endocytosis, enhances cell surface levels of APP and stimulates non-amyloidogenic APP processing [258]. Another member of the LDL receptor family, LR11/SorLA, has also been shown to mediate APP traffic [summarized in ref. 259]. SorLA is believed to promote non-amyloidogenic processing of APP, as suggested by its

reduced expression in the AD brain and the elevated production of Aβ upon ablation of SorLA [260]. Amyloidogenic processing of APP is believed to occur primarily in the endosomal system following internalization of cell-surface APP. SorLA functions as a sorting receptor, and is thought to maintain APP in the Golgi, thereby preventing it from reaching the cell surface and subsequent endocytosis to the endosomes, and thus diverting it from the amyloidogenic pathway [261]. SorLA was later shown to interact directly with APP and BACE and to reduce APP-BACE interactions, suggesting that the reduction of amyloidogenic processing also results from its interference with APP-BACE association [262, 263]. A fourth member of the LDL receptor family implicated in APP traffic is ApoER2, which, like LRP, binds APP and escorts it to lipid rafts; however, unlike LRP, ApoER2 reduces the internalization rate of APP and elevates amyloidogenic processing at the cell surface [264]. F-spondin, an extracellular ligand of both APP and ApoER2, is believed to mediate a ternary complex with these two proteins, leading to enhancement of their respective cell surface levels and metalloprotease-mediated processing [109]. Intriguingly, each of these four transmembrane escort proteins is also cleaved by γ-secretase, which suggests a negative-feedback system, in which the escort protein is enzymatically degraded along with its cargo.

Various cytosolic adaptor proteins – the most prominent of which are members of the X11/Mint and Fe65 families – have been reported to bind the intracellular C-terminal YENPTY motif of APP (important for its traffic) via PTB domains, and thereby modulate processing of APP. Though the precise role of the X11/Mint proteins is presently unknown [reviewed in ref. 265], and controversial reports have been published [266], much data suggest that these proteins, along with synergistic contributions from Alcadeins [267] and Munc18a [268], inhibit production of Aβ, possibly by maintaining APP in an immature state in early compartments of the secretory pathway [269–272]. Recently, a role in APP traffic from the TGN has been established for the γ isoform of X11 [273].

The Fe65 family of adaptor proteins contain two PTB domains, the second of which (PTB2), binds to the APP family of proteins (APP, APLP1, APLP2) [274, 275]. Fe65 and its homolog – Fe65-like (Fe65L) and Fe65L2 – have been reported to enhance Aβ production [276–278]. Although the mechanism by which Fe65 action is exerted is presently unknown, it may occur via concomitant interaction with LDL receptor family members. Through its other PTB domain (PTB1), Fe65 can interact with a variety of proteins, including LRP1 and ApoER2, and can functionally link these two receptors to APP [279–282]. Fe65L1 has also been shown to enhance LRP degradation, suggesting that Fe65 family members may funnel APP and LRP in a tripartite complex toward secretase-containing compartments [283].

Disabled (Dab)-1, another PTB domain-containing protein binds to an NPxY motif in the cytosolic domain of APP, APLP1, APLP2, ApoER2, VLDLr, and LRP [279, 284–286], each of which is a substrate for γ-secretase. Dab1 was reported to increase cell surface levels of APP and ApoER2 and to elevate non-amyloidogenic APP processing [287], though elevated amyloidogenic processing has also been reported [288].

Numerous other adaptor proteins have been reported to bind APP and influence its processing, including c-Jun N-terminal kinase (JNK)-interacting protein-1 (JIP-1b) [289], Pin1 [290], ShcA and ShcC [291–292], PAT1 [293], BRI2 [294], and ARH [295].

Is γ-secretase a processive protease?

Similar to other I-CLiPs [26, 36, 296], γ-secretase cleaves several substrates including APLP-1, APP [103], CD44 [111], and Notch-1 [163], at multiple locations. APP is cleaved intramembranously at the γ-site, located in the center of the TMD near residues 40–42 (using the numbering of Aβ), the ε-site, located at the C-terminal end of the TMD near residue 49 [297], and the ζ-site, located near residue 46 [298]. Investigation into the relationship between these various cleavage sites has demonstrated that Aβ49 is likely the progenitor of Aβ46, and that Aβ46 is an intermediate precursor of Aβ40/42 [299], giving rise to a sequential proteolytic cascade model, in which small ~three residue fragments are consecutively truncated from the C terminus after an initial cleavage near the membrane-cytosol interface [299, 300] (Fig. 7). In agreement with this model, evidence suggests a sequential proteolysis of Notch, in which the S4 cleavage event, occurring near the middle of the Notch TMD, depends upon the S3 cleavage event, occurring near the membrane-cytosol interface [301]. Several studies [299, 300] have noted the orientation of APP cleavage sites on the same side of the transmembrane helix. Indeed, the periodicity of the ε, ζ, and γ cleavage sites is similar to the periodicity of an α helix, suggesting a model in which the C terminus of an α-helical substrate is ratcheted into the catalytic site of γ-secretase, undergoing consecutive cleavage events that release a peptide fragment of approximately three or four amino acids in length.

Substrate specificity of γ-secretase

γ-Secretase is a very versatile protease, having already been reported to cleave nearly 60 unique type I

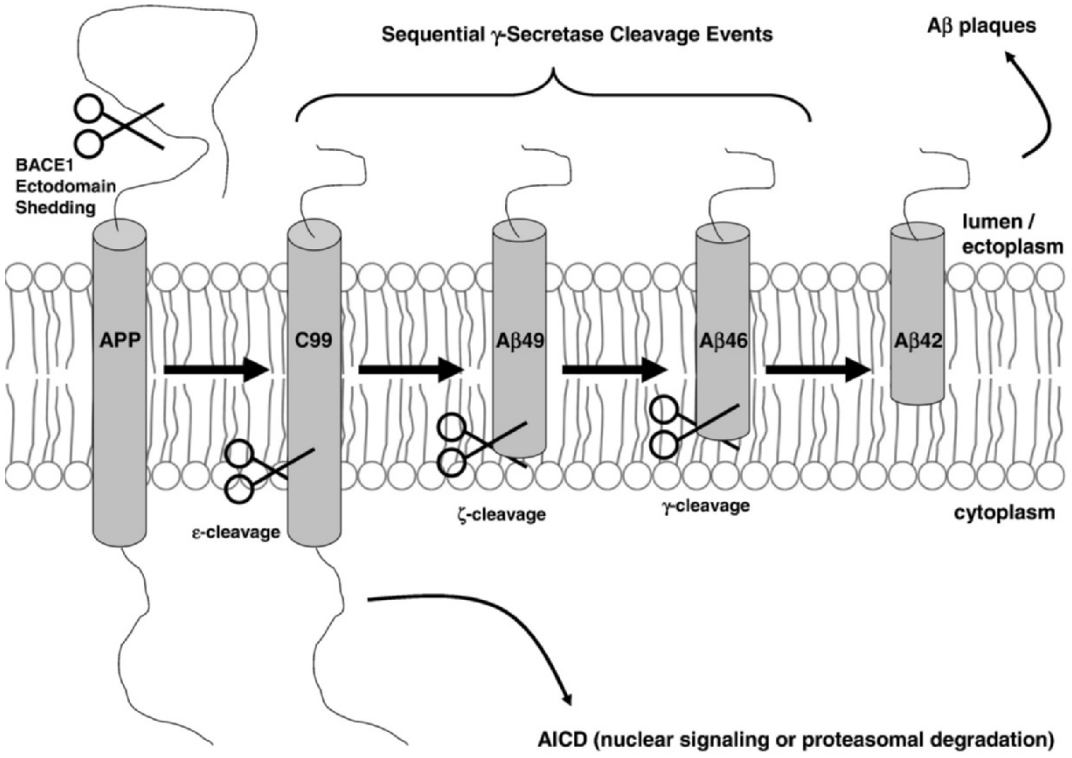

Figure 7. Model for processivity of γ-secretase proteolysis. An initial ectodomain shedding event allows substrate recognition by γ-secretase, which then executes successive juxtamembranous and intramembranous cleavage events. γ-Secretase cleavage often yields a transcriptionally active intracellular domain which can translocate to the nucleus to regulate gene expression.

transmembrane proteins (Fig. 4). Many of its substrates contain domains that are capable of translocating to the nucleus and modulating transcription of various genes [302]. Others do not appear to have a definitive signaling function, leading to γ-secretase's acquisition of the moniker 'proteasome of the membrane' [303]. Potential pharmacological modulation of γ-secretase cleavage of C99 (the precursor to Aβ) [304], considered an important therapeutic target for AD [305], must not interfere with γ-secretases cleavage of its diverse collection of substrates.

Various studies have sought to define characteristics that distinguish γ-secretase substrates from peptides that are not cleaved, e.g., MUC1 [110]. TMD orientation (type I–N-terminal out) and ectodomain length (the average reported ectodomain length for γ-secretase substrates is slightly under 15 amino acids) were thought to be requisite features of all efficiently processed substrates [96]; however, the discovery of peptides contradicting both these principles suggests the importance of other features for substrate recognition by γ-secretase. Though the vast majority of γ-secretase substrates are type I TM proteins, type II TM proteins [306, 307] as well as the glutamate receptor subunit 3 (GluR3), a multipass TM protein, have been reported to undergo PS-dependent processing [308]. Processing has been reported for full-length E-cadherin, which has an extracellular domain of more than 500 residues [132] as well as full-length VEGF receptor [188]. Lastly, γ-secretase does not appear to recognize a scissile bond by TM depth, as it is capable of cleaving at sites throughout the TMD, at the membrane-cytosol interface [132, 135], and in the cytosolic juxtamembranous region [159]. We have conducted an extensive bioinformatic examination of substrate primary, secondary, and quaternary structures to determine structural characteristics that predispose a substrate to γ-secretase proteolysis.

Recognition of substrate primary structure by γ-secretase

Sequence conservation among substrates is minimal, and mutations in substrates are apparently well tolerated by γ-secretase, as evidenced by a number of mutagenesis studies on APP [309–313] and other substrates. Nevertheless, there are examples of single point mutations that entirely abolish γ-secretase cleavage, suggesting that while the enzyme is promiscuous, it requires certain sequence or structural characteristics for proper substrate recognition. Single point mutations of the ErbB4 receptor tyrosine kinase (V673I) and the Notch-1 receptor (V1744G), for example, are known to abrogate proteolysis [139, 314]. In both cases, the mutated valine residue is

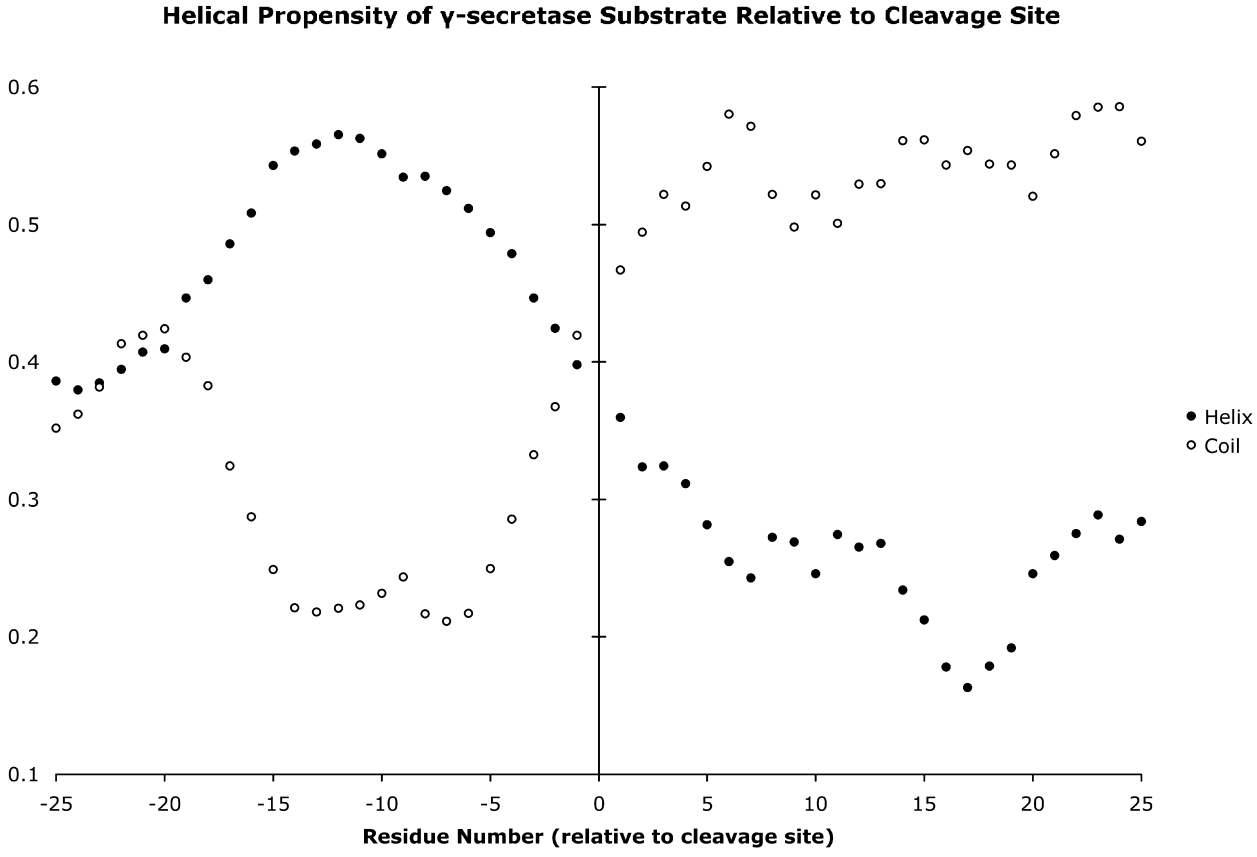

Figure 8. Helical propensities relative to site of cleavage for γ-secretase substrates. Using a combination of methods – *psipred* configured with two different neural networks and *jufo* [315–317] – we compared residues surrounding 23 γ-secretase cleavage sites. These methods perform a three-state prediction, assigning a value to each amino acid indicating the probability that it exists in a random coil state, an α helix, or a β sheet. The results from the three methods were averaged and plotted as a function of residue number relative to the site of cleavage (position 0 in figure).

located immediately adjacent to the site of cleavage. Many γ-secretase substrates contain a similarly positioned valine, which, though critical for the cleavage of Notch-1 and ErbB4, has been demonstrated to be dispensable for the cleavage of other substrates, including APP [309], CD43 [110], and DCC [126]. Hence, the importance of primary structure is equivocal, and recognition likely involves factors at higher levels of protein structure.

Recognition of substrate secondary structure

Proteolysis by other I-CLiPs is enhanced by and often requires helix-destabilizing residues. To determine if a similar requirement exists for γ-secretase proteolysis, we performed secondary structural predictions on all substrates with known primary cleavage sites (Fig. 8). The results suggest that helix destabilization may be important for presenilin-mediated proteolysis. To our knowledge, this is the first such demonstration of the potential relevance of transmembrane helix destabilization for γ-secretase proteolysis. Cleavage appears to most commonly occur near the C-terminal end of the TM helix just inside the membrane-cytosolic interface (Fig. 4).

Recognition of substrate higher-order structure by γ-secretase

A number of γ-secretase substrates – syndecan-3 [195], E-cadherin [318], N-cadherin [319], ErbB4 [320], APP [321], PTPκ, and PTPμ [322] – dimerize via TMD interactions. Several substrates (ErbB4, IGF-1R, CSF-1R, VEGF-1R, EphB2) are receptor tyrosine kinases (RTKs), which are known to dimerize in response to ligand binding [323]. Mutationally induced promotion of APP homodimerization resulted in substantial elevation of Aβ production, suggesting that dimerization may be a direct regulatory factor in the proteolytic processing of APP [193]. In addition to ectodomain dimerization interfaces, a particular motif within the C99 TMD – GxxxG – mediates homodimerization, and mutations affecting the dimerization strength of this motif alter γ-secretase cleavage precision, but not efficiency. Interestingly, more than 25 % of γ-secretase substrates contain the GxxxG

pentapeptide – a canonical TM dimerization motif [324–325] – in their TMD (cf. Fig. 4). Inhibition of C99 dimerization by mutating its GxxxG motifs was shown to specifically reduce production of longer forms of Aβ (e.g., Aβ42) and increase production of shorter forms of Aβ (e.g., Aβ35, Aβ38) [321]. These observations have led to an expansion of the processive proteolysis model proposed by Qi-Takahara, such that PS proteolysis removes consecutive fragments from the exposed C-terminus of APP until cleavage reaches a certain distance from the dimerization motif, whereupon cleavage terminates and Aβ40/42 is released. Disruption or attenuation of the dimerization interface allows continued proteolysis of APP, with the concomitant release of shorter Aβ fragments. According to this model, the terminal cleavage point is determined by the location and strength of the TM dimerization interface [321]. Note that oligomerization-dependent control of proteolysis is unlikely to apply to all γ-secretase substrates, as demonstrated for the Notch receptor [165], suggesting the existence of multiple proteolytic regulatory mechanisms.

Perspectives

Common themes have begun to emerge among the evolutionarily diverse class of proteases known as I-CLiPs. A regulated event such as ligand binding or ectodomain shedding precipitates complex subcellular traffic events or movement between membrane microdomains, following which the formerly separated protease and substrate come into contact. Often, the proteolytic cleavage that ensues is not merely degradative in nature, but initiates a signaling cascade that can impact genomic expression, communicate information to other cells, effect cellular homeostasis, or nucleate pathological events. These peculiar proteases have co-opted classical protease motifs. Their sequence specificity is typically quite loose, and factors beyond the primary level of protein structure, particularly TM helix destabilization, appear more important for substrate recognition and proteolysis.

Although much has been learned, many basic questions remain unanswered. The substrate/enzyme stoichiometry, relevance of additional cofactors, and subcellular localization and traffic of γ-secretase remain ill-defined. The traffic between subcellular compartments and membrane microdomains for C99 and other γ-secretase substrates – a critical facet of proteolytic regulation – requires much additional investigation. While correlations have been reported, the discovery of causal factors regulating substrate traffic and ectodomain shedding has been limited. Indeed, for C99 it remains unclear which trafficking and/or enzymic properties control the rate of Aβ production. The processive model of intramembranous cleavage also remains highly suppositional. Such basic issues require continued attention to acquire a deeper understanding of the various physiological and pathophysiological events mediated or influenced by γ-secretase proteolysis. The clinical utility of agents that modulate γ-secretase activity, considered a viable therapeutic avenue for the prevention and treatment of AD, will be enhanced by an elucidation of these fundamental questions.

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