

Intramembrane-cleaving aspartic proteases and disease: presenilins, signal peptide peptidase and their homologs

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Recent studies demonstrate that presenilins (PSs) and signal peptide peptidase (SPP) are members of a novel protease family of integral membrane proteins that may utilize a catalytic mechanism similar to classic aspartic proteases such as pepsin, renin and cathepsin D. The defining features of the PSs and SPP are their ability to cleave substrate polypeptides within a transmembrane region, the presence of two active site aspartate residues in adjacent membrane-spanning regions and a conserved PAL motif near their COOH-terminus. PSs appear to be the catalytic subunit of multiprotein complexes that possess γ -secretase activity. Because this activity generates the amyloid β peptide (A β) deposited in the brain of patients with Alzheimer's disease (AD), PSs are considered therapeutic targets in AD. In contrast to PSs that are not active unless part of a larger complex, SPP does not appear to require protein co-factors. Because of its requirement for hepatitis C virus maturation and a possible immune modulatory role, SPP is also considered a potential therapeutic target. Four additional PS/SPP homologs have been identified in humans; yet, their functions have not been elucidated. Herein, we will review the recent advances in our understanding of the PS/SPP family of proteases as well as discuss aspects of intramembrane cleavage that are not well understood.

INTRODUCTION

In order to more accurately reflect their catalytic activities, proteases have been referred to as peptide peptidohydrolases. This terminology reflects the catalytic mechanism in which a water molecule plays an essential role in cleaving a peptide bond. Until recently, all characterized 'classic' proteases had been shown to have an active site domain located in the aqueous environment of the cytoplasmic, luminal or extracellular space. Thus, cleavage of peptide bonds buried within the hydrophobic interior of a cellular membrane was postulated to require either breakdown of the membrane or transport of substrate out of the lipid bilayer to an aqueous environment. The recent identification of several families of membrane proteases that seem capable of cleaving peptide bonds present within the lipid bilayer has dramatically changed the concept of how transmembrane regions of proteins may be cleaved (1,2).

Three families of proteases that carry out 'intramembrane proteolysis' are recognized (1,2). Though exact catalytic mechanisms remain elusive, there is evidence that the active

site of these proteases could lie within the plane of the lipid bilayer, and that proteases within each family may utilize conserved catalytic mechanisms analogous to those found in classic proteases. The first family, whose prototypic member is the human site-two protease (S2P) that cleaves and activates sterol regulatory element binding proteins (SREBPs), appears to be a group of metalloproteases (3). The second family, the rhomboids, whose prototypic member *Drosophila* Rhomboid-1 cleaves and liberates several EGF ligands, appears to be a group of serine proteases (4). A third family, whose prototypic members are the presenilins (PSs) involved in cleavage of the amyloid β protein precursor (APP) and Notch, appears to be a group of aspartic proteases (5,6). Collectively these proteases have been referred to as intramembrane-cleaving proteases (I-CLiPs). For several reasons a great deal of attention has focused on the aspartic I-CLiP family. First, the prototypic members of this family, the human PSs, are therapeutic targets in Alzheimer's disease (AD). Second, a new member of this family, signal peptide peptidase (SPP), was recently identified and shown unequivocally to possess proteolytic activity and have

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biological functions that may also make it a therapeutic target. Herein, we will discuss these recent advances with respect to the presenilin/signal peptide peptidase family of I-CLiPs.

PRESENILINS

PSs are integral membrane proteins with 6–8 predicted transmembrane regions. They were first identified through genetic linkage analysis in families with early onset autosomal dominant forms of AD (7,8). PSs are synthesized as ~50 kDa proteins, but are rapidly endoproteolyzed into NH₂- (~30 kDa) and COOH- (~20 kDa) terminal fragments, which remain associated along with other proteins in a high molecular weight complex (9,10). To date, ~100 AD-linked mutations have been found in presenilin 1 (PS1) and several more in presenilin 2 (PS2) (11). Almost concurrent with the identification of mutations in PSs as genetic causes of AD, there was some evidence linking PSs to the proteolytic activity referred to as γ -secretase (12). This activity is involved in the production of the amyloid β peptide (A β) from its precursor, APP. Following cleavage of APP by β -secretase, γ -secretase cleaves the membrane bound COOH-terminal APP fragment within its transmembrane region generating both the ~4 kDa A β peptide and a cognate COOH-terminal cleavage product. The cut in the membrane is heterogeneous with multiple A β species produced ranging in length from 32–42 amino acids (13). The most prevalent form of A β is one of 40 amino acid residues (A β 40); under typical circumstances A β 40 represents >50% of the A β secreted from a cell. However, an A β species of 42 amino acids (A β 42) appears to play a causal role in AD pathogenesis. To date, all AD-linked mutations in PS1 and PS2 have been shown to increase the relative production of A β 42 even in asymptomatic carriers, and expression of a mutant PS gene in transgenic mice overexpressing human APP, accelerated accumulation and aggregation of A β in the brain (12,14).

Despite the evidence that mutations in PSs altered the γ -secretase activity that generated A β , no one readily endorsed the idea that PSs were γ -secretases. PSs did not bear homology to classic proteases; thus, it was proposed that PSs influenced γ -secretase activity indirectly. Over the past few years, genetic and biochemical evidence has emerged demonstrating that PSs are in fact γ -secretases. Knockout studies of PS1 and PS2 demonstrated an essential role of these proteins in γ -secretase cleavage. PS1 knockout decreased γ -secretase activity by ~80%, a PS2 knockout had little or no effect, and a combined PS1 and PS2 knockout abolished γ -secretase activity (15–18). Biochemical studies showed that PS1 co-fractionates with γ -secretase activity in a high molecular weight complex and this *in vitro* activity could be recovered in the pellet after immunoprecipitation with antibodies to PS1 (19). Significantly, mutation of conserved aspartate residues (D257 and D385 in PS1, and D263 and D366 in PS2) present in two adjacent transmembrane regions produced dominant negative forms of PS that inhibited γ -secretase activity in cells (5). These studies together with inhibitor studies suggested that PSs might be aspartic type proteases (20). Finally, inhibitors of γ -secretase activity have been shown to directly target both PS1 and PS2 (21–24). Moreover, given that two groups had specifically designed their inhibitors to be transition state analogs of

aspartic proteases (21,22), it certainly appears that despite the lack of demonstrable homology to known aspartic proteases, PS may be just that.

Recent insight into the other components of the high molecular weight complex that contains γ -secretase activity and PSs, now offers a more complete picture of γ -secretase. Three additional proteins are required for γ -secretase activity in cells. Nicastrin (Nct, APH-2) is a ~130 kDa type I membrane protein that was originally purified from the complex (25). Two additional proteins, APH-1, another integral membrane protein of ~30 kDa with multiple transmembrane regions, and PEN-2, a small hairpin like protein of ~12 kDa, were initially isolated from genetic screens in *Caenorhabditis elegans* (26). Together with a PS, the three components appear to comprise a minimal functional γ -secretase complex (27–29). Although the precise function of each subunit is not yet definitively established, it appears that PSs are the catalytic component, and that Nct, APH-1 and PEN-2 play various roles in the stabilization and maturation of the complex. It is also possible that these 'accessory' proteins are involved in substrate presentation.

The biological activities of γ -secretase and PSs have been extensively reviewed (2,30). Although some activities of PSs are distinct from their protease activity (e.g. stabilization of β -catenin), many are not. γ -Secretase activity regulates Notch and APP signaling by promoting the release of the cytoplasmic tail from a membrane bound fragment of each precursor. It is not clear whether the release of small peptides, such as A β , into the exoplasmic space has any demonstrable physiologic function. It is also not clear whether γ -secretase cleavage of substrates other than Notch and APP has some physiologic functions. It is possible that γ -secretase cleavage of certain substrates may simply be a mechanism to breakdown these proteins. A current list of γ -secretase substrates, cleavage products and putative functions are listed in Table 1.

SIGNAL PEPTIDE PEPTIDASE

The second, more recently identified member of the aspartic I-CLiP family is the SPP of higher eukaryotes (31). Typical for an aspartic I-CLiP, SPP is a predicted multispreading membrane protein containing two putative active site aspartates that are located in the centre of adjacent transmembrane regions. The latter feature is consistent with the observed cleavage of substrate peptides in the centre of their membrane-spanning portion. In addition, SPP contains two endoglycosidase H-sensitive N-glycans and an ER retrieval signal, KKxx, ascribing SPP a function in the endoplasmic reticulum (ER) (31).

The role of SPP in cell function is poorly understood. The SPP substrates identified to date, represent a variety of signal peptides and a viral protein (Table 2) (31–34). They do not unequivocally demonstrate to a role of SPP either in the degradation of signal peptides that may otherwise accumulate in the ER membrane, or in the activation of signaling or regulatory molecules, or both. Potential orthologs of SPP are found in the genomes of animals and plants but not fungi and bacteria (31,35). Therefore, the prime role of SPP may not necessarily be the clearance of membranes from signal peptides, which are produced in all organisms, and a more basic mechanism may exist to fulfil that function. We may thus

Table 1. PS/ γ -secretase substrates

| Substrate | Relevant fragments | Functions |
|--------------------|--|--|
| APP | A β and cytoplasmic domain (AID, AICD) | AID: Nuclear signaling in complex with TIF; regulation of ER calcium stores. A β : physiologic role unknown, pathologic role in AD |
| APLP1 | Cytoplasmic domain | Nuclear signaling? |
| APLP2 | Cytoplasmic domain | Nuclear signaling? |
| Notch 1-4 | Cytoplasmic domain (NICD) | Nuclear signaling in complex with transcription factor. |
| Erb-B4 | Cytoplasmic domain (S80) | Signaling by translocation of kinase domain to nucleus. |
| E-Cadherin | Cytoplasmic domain | Regulation of adherens junction? |
| LRP | Cytoplasmic domain | Unknown |
| Delta-1 and Jagged | Cytoplasmic domain | Antagonist role in Notch signaling? |
| Nectin-1 α | Cytoplasmic domain | Unknown |
| CD44 | Cytoplasmic domain | Signaling? |

speculate that SPP, in analogy to other I-CLiPs, may promote the liberation of bioactive peptides or proteins from the ER membrane. The generation of HLA-E epitopes in humans represents the first example of such an SPP-dependent production of a bioactive peptide, which in this case functions as a reporter. HLA-E epitopes that play a key role in immune surveillance, are produced from the signal sequences of the antigen presenting MHC class I molecules in a process that depends on SPP (36). The epitopes are eventually presented at the cell surface of most of our cells, where they are recognized by the natural killer (NK) cells of the immune system. Presentation of these signal peptide-derived epitopes is an indirect indication that the probed cell is healthy and had properly synthesized MHC class I molecules. If such epitope presentation is defective or otherwise disturbed, e.g. due to a virus infection or transformation to a tumor cell, these impaired cells are thought to be recognized and eliminated by the NK cells (37).

The substrate spectrum of SPP may not be restricted to classic signal peptides, which typically are short, NH₂-terminal extensions of a precursor protein. The finding that SPP is exploited by the hepatitis C virus (HCV) for the processing of the viral core protein, implies that the protease may also catalyze intramembrane cleavage of membrane anchored proteins, similar to other I-CLiPs (34). During biosynthesis of viral proteins in HCV infected cells, the immature HCV core protein is transiently anchored in the ER membrane via a COOH-terminal, signal peptide-like sequence. Intramembrane proteolysis by SPP promotes the final processing of core protein and its release from the ER membrane into the cytosol. Likewise, we may speculate that proteins anchored in the ER membrane by a signal peptide-like sequence, may be liberated from the ER membrane and activated upon cleavage by SPP.

PRESENILIN AND SPP: SIMILARITIES AND DIFFERENCES

Despite limited areas of direct sequence homology, PSs and SPP are membrane proteins whose sequences can be aligned

Table 2. SPP substrates

| Substrate | Relevant fragments | Functions |
|--|---------------------------------------|--|
| Signal peptides of human MHC class I molecules | HLA-E epitope | Immune surveillance |
| Signal peptide of prolactin | Cytoplasmic portion of signal peptide | Calmodulin signaling? (enhancer of prolactin secretion?) |
| Hepatitis C virus polyprotein | HCV core protein | Subunit of virus capsid |

almost throughout their entire lengths (31,35). PSs and SPP share identical active site motifs, YD and LGLGD (Table 3). In addition, they contain a third conserved region, PAL, which seems characteristic for the entire aspartic I-CLiP family. Moreover, the YD and LGLGD motifs, which contain the catalytic aspartate residues, are unusual in that they are present within predicted adjacent and opposing transmembrane regions. The similarities between PSs and SPP, particularly the identity of the active site motifs, point to a common catalytic mechanism. Indeed, a series of protease inhibitors including aspartic protease transition state analogues, targeted to the active site of SPP and PSs, inhibited both activities (38). Because both enzymes are potential targets for therapeutic intervention—PSs for the treatment of AD and SPP for the anti HCV therapy—it will be a challenging task for the future to define compounds and conditions for the selective inhibition of either protease.

Another common feature, and one that is typical of many I-CLiPs, is that both PS and SPP activity appear to require prior cleavage of the substrate to remove its ectodomain. SPP substrates are first cleaved by signal peptidase (33), whereas PS/ γ -secretase substrates are first cleaved by shedding proteases (e.g. ADAM family members and β -secretase) (39–41). In most cases studied to date, this initial cleavage is essential for the intramembrane cut to occur. Thus, substrate specificity of PS/ γ -secretase and SPP may be determined as much by this priming cleavage as the respective substrate sequence itself.

One of the most striking differences between PSs and SPP is the apparent opposite positioning of the active site motif-containing transmembrane regions within the plane of the membrane (31,42). This positioning correlates with the opposite orientation of the respective substrates. PS substrates have a type I membrane topology with their COOH-termini facing the cytosol, whereas SPP substrates have a type II topology with their NH₂-termini facing the cytosol. Thus, it appears that related proteases with inversed proteolytic domains evolved for the cleavage of membrane spanning peptides of different topologies.

Despite the common features, there are major differences between PSs and SPP. The reconstitution of human SPP in the yeast *Saccharomyces cerevisiae*, which does not express an orthologous 'signal peptide peptidase', suggested that human SPP does not require additional proteins for activity (31). In contrast, PSs appear to require at least three additional protein partners for activity. PSs undergo endoproteolytic activation, which may depend on these components and, together

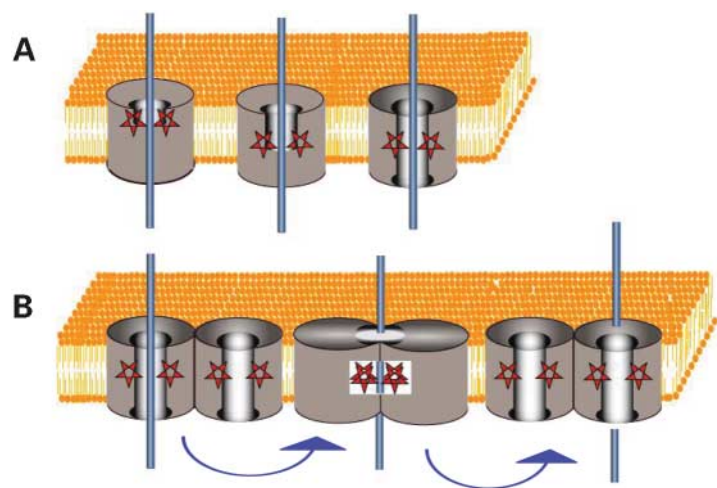


Figure 1. Possible models of cleavage by an I-CLiP. Several models are presented that could permit water access to the active site. **(A)** An I-CLiP may form a shallow or deep hydrophilic pocket in the membrane. This would allow access of water to the catalytic residues (indicated by the two stars). The pocket may form upon a conformational change induced by binding of the substrate. **(B)** Two units of an I-CLiP may act as a dimer to carry out the cleavage. In this case, substrate binding to one I-CLiP unit may induce a conformational change and the formation of a pore containing a merged active site.

Table 3. PS and PSH family members

| Protein | Chr. locus | Protein length | Asp 1 | Asp 2 | PAL | Endoproteolyzed | PA domain | Function |
|-------------|------------|----------------|-------|---------|--------|-----------------|-----------|--------------------------|
| PS1 | 14 | 467 | YDLC | LGLGDFI | LPALPI | Yes | No | γ -Secretase |
| PS2 | 1 | 446 | YDLV | LGLGDFI | LPALPI | Yes | No | γ -Secretase |
| SPP | 20 | 377 | YDVF | LGLGDVV | QPALLY | No | No | Signal peptide peptidase |
| SPPL3/PSH1 | 12 | 385 | YDVF | LGIGDIV | QPALLY | ? | No | Unknown |
| SPPL2a/PSH5 | 15 | 409 | YDVF | LGFGDII | QPALLY | ? | Yes | Unknown |
| SPPL2b/PSH4 | 19 | 564 | YDIF | LGFGDIL | QPALLY | No | Yes | Unknown |
| SPPL2c/PSH2 | 17 | 684 | FDVF | LGFGDIV | QPALLY | No | Yes | Unknown/pseudogene? |

with these additional proteins, forms an active γ -secretase complex. Such activation and complex formation may provide means of control of the protease’s intramembrane-cleaving activity, and regulate substrate delivery and/or trafficking to the intracellular site of action. In the case of SPP, there is no indication for endoproteolysis or any another activation step. Nevertheless, its activity is almost certainly regulated in some fashion; SPP does not appear to randomly attack membrane proteins in the ER. Rather than the protease activity, properties of the substrates may account for controlled proteolysis by SPP (33).

OTHER HOMOLOGS

Shortly after the identification of human PSs, homologs were recognized in plants, invertebrates and vertebrates. Some of the close homologs such as those found in *C. elegans* and *Drosophila*, have been extensively studied and shown to function as aspartic I-CLiPs in high molecular weight complexes like the human PSs (reviewed in 2). More recently, in addition to SPP, a number of other potential aspartic I-CLiPs with less obvious homology to PSs have been recognized by database searching (Table 3) (31,35,43). These proteins have no identified function and have been referred to by various names. Herein, they will be referred to as presenilin

homologs/signal peptide peptidase-like proteases (PSH/SPPL), although with future functional assignment we propose that they would be renamed to more accurately reflect their function. In humans, four PSH/SPPLs with unknown functions have been recognized. PSH/SPPLs are predicted to be integral membrane proteins with multiple membrane spanning regions, and to contain both the conserved transmembrane aspartates in the motifs YD and LGhGD, and the PAL motif near the COOH-terminus. In addition, several members of the family contain a protease-associated domain near their NH₂-terminus. When found in some classic protease, this domain is involved in substrate binding (44).

MODELS OF CLEAVAGE

Although much has been learned about intramembrane cleavage, the exact manner in which these reactions occur remains a mystery. The conservation of putative active site motifs between I-CLiPs and classic proteases indicates that the catalytic mechanism of I-CLiPs most likely involves hydrolysis. If this is the case, then water must somehow gain entry into the active site of these proteases and the labile bonds within the substrate’s transmembrane region must at some point be exposed to an activated water molecule. In Figure 1,

theoretical models that might fulfill these two requirements are shown. The first model (Fig. 1A) is one in which the multiple transmembrane regions of the protease form a hydrophilic pocket or groove at one face of the membrane. This model is compatible with I-CLiP cleavage sites that lie close to the cytosolic or luminal face of the membrane, such as those that release the cytoplasmic tail of APP and Notch. However, cleavage events such as those that generate A β and appear to occur in the middle of the membrane, might either require some perpendicular movement of the substrate or protease within the membrane, or possibly a very deep hydrophilic pocket. A variant of this model might be that the transmembrane regions of the I-CLiP adopt a horseshoe like structure. Upon association of the substrate to the active site, a channel or pore might be formed allowing access of water to the site of hydrolysis. Such a model might require distinct initial binding sites of the substrate followed by transfer into the active site. Another model (Fig. 1B), that might be suggested by recent data showing that both PSs and SPP may form dimers *in vivo*, is one in which the initial binding of substrate occurs in one active site. This binding could then induce a conformational shift resulting in the merger of the two subunits that together form a pore like structure. Alternatively, a distinct substrate-binding site on one subunit could result in presentation of the substrate to the active site of the other subunit. Additional models of cleavage that combine various aspects of these models are possible. Finally, it is possible that a given I-CLiP may utilize a novel mechanism of catalysis that is compatible with cleavage of a peptide bond in a lipid environment.

CONCLUSIONS

The aspartic I-CLiPs represent a newly recognized and biomedically important family of proteases. Intense study of PSs and γ -secretase has led to the development of potent inhibitors of γ -secretase activity several of which have entered phase I trials in humans. Distinct γ -secretase inhibitors have overlapping activity against SPP (38), it is highly expected that many of those compounds may also target the other PSH/SPPLs. Thus, the specificity of γ -secretase inhibitors must be questioned. Because of the potential clinical significance of γ -secretase inhibitors, there is urgent need to increase our understanding of these proteins. Even with the experience gained by studying PSs and SPP, this will remain a difficult task. To date, there is no easily identifiable strategy that is assured of identifying function or substrate for these novel aspartic I-CLiPs. Nevertheless, because inhibitor specificity may prove instrumental, if inhibitors of aspartic I-CLiPs prove to be of therapeutic utility, this assignment of function and development of activity assays for each is extremely important.

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