A C-terminal Region of Signal Peptide Peptidase Defines a Functional Domain for Intramembrane Aspartic Protease Catalysis*5

Saravanan Kumar Narayanan, Toru Sato, and Michael S. Wolfe1

From the Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

Intramembrane proteolysis is now firmly established as a prominent biological process, and structure elucidation is emerging as the new frontier in the understanding of these novel membrane-embedded enzymes. Reproducing this unusual hydrolysis within otherwise water-excluding transmembrane regions with purified proteins is a challenging prerequisite for such structural studies. Here we show the bacterial expression, purification, and reconstitution of proteolytically active signal peptide peptidase (SPP), a membrane-embedded enzyme in the presenilin family of aspartyl prostases. This finding formally proves that, unlike presenilin, SPP does not require any additional proteins for proteolysis. Surprisingly, the conserved C-terminal half of SPP is sufficient for proteolytic activity; purification and reconstitution of this engineered fragment of several SPP orthologues revealed that this region defines a functional domain for an intramembrane aspartyl protease. The discovery of minimal requirements for intramembrane proteolysis should facilitate mechanistic and structural analysis and help define general biochemical principles of hydrolysis in a hydrophobic environment.

Intramembrane-cleaving proteases (I-CLiPs)2 are a family of polytopic enzymes that catalyze hydrolysis of substrate transmembrane domains. Although these proteases bear essentially no sequence similarity to their water-soluble counterparts, they have apparently converged on similar catalytic mechanisms. Thus, the I-CLiPs include the Site-2 protease family of putative metalloprostases, the Rhomboid-type serine prostases, and presenilin and presenilin-like aspartyl prostases (1). Such mechanistic categories have been assumed from mutagenesis studies and the types of compounds that inhibit these prostases. However, the first structures of an I-CLiP, the Escherichia coli Rhomboid GlpG, have recently been reported, confirming the presence of a serine-histidine catalytic dyad 10 Å within the membrane and the categorization of Rhomboid as a bona fide intramembrane aspartic protease (2–4).

The new reports on the structure of Rhomboid emphasize the point that biochemical studies of I-CLiPs are prerequisites for structure determination and the mechanistic interpretation of such structures. Toward this end, tremendous efforts have focused on presenilin and the γ-secretase complex, a putative membrane-embedded aspartyl protease that produces the amyloid-β protein implicated in Alzheimer disease and that plays a key role in Notch receptor signaling during cell differentiation (5). Although presenilin is apparently the catalytic component, it must assemble with three other membrane protein cofactors and be endoproteolyzed into two pieces to form active γ-secretase (6–8). The entire complex has been purified in active form, allowing the determination of low resolution structures by electron microscopy and single particle analysis (9, 10). However, the challenge of co-crystallizing presenilin with its cofactors makes structural determination of the γ-secretase complex at the atomic level quite difficult.

In contrast to the complexity of γ-secretase, a family of presenilin homologues appears more suitable for detailed structural analysis. The most studied is signal peptide peptidase (SPP), which cleaves remnant signal peptides left behind in the membrane by the action of signal peptidase and also plays key roles in immune surveillance and the maturation of certain viral proteins (11). The ability to reconstitute SPP activity in Saccharomyces cerevisiae, a eukaryotic organism that does not encode this protease, by expressing the presenilin homologue alone suggested that other protein cofactors are not required (12). The chemistry behind proteolysis by SPP and presenilin is presumably the same (i.e. both are membrane-embedded aspartyl prostases), and a number of γ-secretase inhibitors and modulators can likewise affect SPP activity (13). Thus, understanding SPP may provide insight into the workings of presenilin within the γ-secretase complex. Here we show that SPP from several species can be expressed in bacteria and purified in proteolytically active form, formal proof that this protein works alone. Surprisingly, a conserved C-terminal region of SPP is sufficient for enzymatic activity, suggesting that it represents a functional domain of this aspartyl I-CLiP. These studies provide important insights into the nature of intramembrane proteolysis and

*This work was supported by Grants AG17574 and AG15379 (to M. S. W.) and a Harvard Medical School Lefler fellowship (to S. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1To whom correspondence should be addressed: Center for Neurologic Diseases, Brigham and Women’s Hospital, Harvard Medical School, 77 Ave. Louis Pasteur, H.I.M. 754, Boston, MA 02115. Tel.: 617-525-5511; Fax: 617-525-5252; E-mail: mwolfe@rics.bwh.harvard.edu.

2The abbreviations used are: I-CLiP, intramembrane-cleaving protease; SPP, signal peptide peptidase; Prl, prolactin; DDM, dodecyl-β-o-maltoside; TM, transmembrane; PC, phosphatidylycholine; PE, phosphatidylethanolamine; CHO, Chinese hamster ovary; MBP, MBP-binding protein.
set the stage for detailed structural analysis through crystallography, NMR, and other biophysical approaches.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Plasmid encoding human SPP was constructed by amplifying the human SPP gene from the previously constructed SPP-CTV5his plasmid (14). The resultant PCR product was ligated into the pMal-p2x vector (New England Biolabs). *Schizosaccharomyces pombe* haploid wild type strain *(hura4-d18 leu1-32)* was obtained as a gift from Dr. Dieter Wolf at the Harvard School of Public Health. The *S. pombe* genomic DNA was extracted from spheroplasts using a DNeasy Blood and Tissue kit (Qiagen) and used as a template for SPP gene amplification, and the amplified gene was inserted into the vector. *Drosophila* SPP and SPPL3 genes were inserted into the pMal-p2x vector using the same strategy as described above. PCR products of these genes were amplified using expressed sequence tags (Open Biosystems) as templates. *Drosophila* SPP was also subcloned into the pET22b vector (Novagen) to introduce a His$_6$ tag at the 3' prime position for detection and isolation.

Functional domains of human, *S. pombe*, and *Drosophila* SPPs were subcloned into both pET22b and pMal-p2x vectors using the same strategy as described above. In these cases, His$_6$ tag was introduced in both pMal-p2x and pET22b constructs at the 3' position. Mutation of the aspartate codons was performed using QuikChange II site-directed mutagenesis kit (Stratagene).

**Protein Expression and Membrane Isolation**—The plasmids containing the genes of interest were transformed into the C43 (DE3) strain of *E. coli*. Cultures were grown in LB media with ampicillin, shaken at 37 °C until an optical density of 0.8 was reached. At this point, 0.3 mM isopropyl 1-thio-β-D-galactopyranoside was added, and the culture was grown at 23 °C for 12–14 h. Harvested cells from 400 ml of culture were resuspended in 20 ml of PSGS buffer (25 mM phosphate, 100 mM NaCl, 8% glycerol, 5% sucrose, pH 7.5) containing 1 μg/ml DNase-I and a protease inhibitor mixture (Roche Applied Science). The cells were mechanically disrupted and centrifuged at 10,000 × g for 15 min to remove debris, and the supernatant was centrifuged at 100,000 × g for 1.5 h to isolate the membranes. Membranes were washed in PSGS and centrifuged again at 100,000 × g for 1 h.

**Purification of SPP**—The isolated bacterial membranes were solubilized in 1% n-dodecyl-β-D-maltoside (DDM) in PSGS containing a protease inhibitor mixture and rocked at 4 °C for 2 h. The supernatants were centrifuged at 100,000 × g for 1 h to yield solubilized membrane fractions containing the expressed SPP. These fractions were diluted, and SPP was affinity-purified using either amylose resin (New England Biolabs) (for MBP fusion proteins) or talon resin (BD Biosciences) (for His$_6$-tagged proteins). Bound protein was either eluted or kept on the beads for the activity assay. The elution buffer (0.1% DDM in PSGS) contained 25 mM maltose to release MBP fusion protein or 300 mM imidazole to release His$_6$-tagged protein. The purity and identity of eluted proteins were assessed by Western blotting and by silver and Coomassie staining. Anti-penta-His (Qiagen) and anti-MBP antibodies (New England Biolabs) were used for Western blot detection. Blue native gel (4–16%, Invitrogen) was also used for characterization of eluted proteins.

**Reconstitution of SPP**—Chloroform-solubilized lipids were evaporated to dryness under vacuum. The resulting thin films were hydrated using 1% DDM in phosphate-buffered saline and sonicated to make a 1% suspension of lipids. On-column reconstitution of activity was performed immediately after the isolation of MBP-SPP on amylose beads by adding 0.1% final concentration of the appropriate lipids. Off-column reconstitution was performed immediately after elution. The eluted protein was mixed with sonicated lipids (~1,500,000 of protein: lipid) and kept at 4 °C for 3 h. The resulting proteoliposomes were rapidly diluted, centrifuged at 100,000 × g for 1 h, and used for activity assays. Western blot and quantification were also performed on proteoliposomes.

**In Vitro SPP Activity Assay**—A standardized in vitro cleavage assay was used throughout this study as described (15). Authenticity of SPP activity was assessed in part by incubating the reaction mixture in the presence of an SPP-specific inhibitor, (ZLL)$_2$ ketone (Calbiochem). (ZLL)$_2$ ketone and helical peptide inhibitors were used at the concentration of 10 μM for standard reactions. Helical peptides were designed based on the SPP substrate calreticulin. 5 μM pepstatin A (Sigma), 100 μM diisoucamarin (Sigma), 10 μM E-64 (Sigma), or 10 μM bestatin (Sigma) were used to characterize the sensitivity of SPP activity to inhibitors directed to major protease classes. (Hydroxethyl)urea-type transition-state analog inhibitors were used as described previously (16).

**Glycerol Velocity Gradient**—Globular proteins (albumin from chicken egg white, M, ~45 kDa; bovine serum albumin, ~66 kDa; phosphorylase-b, ~97.2 kDa; alcohol dehydrogenase from *S. cerevisiae*, ~141 kDa; catalase, ~230 kDa) were purchased from Sigma-Aldrich and used as standards. ~2 μg of purified, proteolytically active MBP-SPPs in 1 ml of assay buffer (0.1% foscholine in 25 mM phosphate, 25 mM NaCl, pH 7.5, containing a protease inhibitor cocktail) were loaded into 10 ml of 5–32% linear glycerol gradient-containing assay buffer. Gradients were centrifuged for 15.5 h at 36,000 rpm and 4 °C using an SW41Ti rotor and collected by 0.5-ml fractions. Collected fractions were resolved using 4–20% Tris-glycine SDS-PAGE electrophoresis and stained using Imperial Stain (Pierce).

**RESULTS**

**Expression and Characterization of Active Signal Peptide Peptidases in *E. coli***—Signal peptide peptidases were shown to functionally express in yeast and mammalian cells as N-glycosylated proteins (12, 14, 17). Mutagenesis studies have demonstrated that glycosylation is not essential for proteolytic function, suggesting that expression of active protease in bacteria might be possible (18). Four different variants were expressed in *E. coli* strain C43: SPP from *Homo sapiens*, SPP from *S. pombe*, and SPP and SPP-like protein 3 (SPPL3) from *Drosophila melanogaster*, each as an N-terminal MBP fusion protein containing the PelB leader sequence for the proper insertion

---

3 T. Sato, A. Kuppanna, S. Narayanan, C. Cheng, and M. S. Wolfe, unpublished results.
Intramembrane Aspartic Protease Functional Domain

FIGURE 1. Expression in E. coli of SPPs from several organisms provides active protease. a, design of the MBP-SPP fusion protein. Conserved aspartates and PAL domain are indicated by circles and rectangles, respectively. b, DDM-solubilized SPPs (human SPP, 5, pombe SPP, and Drosophila SPP and SPP-like protein 3) cleave Prl signal peptide sequence. Proteolytic activity was inhibited by (ZLL)\textsubscript{2} ketone. 0, 90, and ZLL represent the assay performed at 0, 90, and 90 min with [ZLL], ketone, respectively. DDM-solubilized membranes expressing MBP alone showed no activity. c, mutating either aspartate to alanine drastically reduced the activity of Drosophila SPP, WT, wild type. d, inhibitors of different classes of proteases showed no effects on Drosophila SPP activity, whereas [ZLL], ketone reduced activity substantially. e, expression of Drosophila SPP-His\textsubscript{6} (without MBP fusion) resulted in an ~40-kDa band as detected with anti-penta-His antibodies in solubilized membranes. f, DDM-solubilized Drosophila SPP-His\textsubscript{6} possesses activity. The observed cleavage product co-migrated with the characterized Prl cleavage product formed from solubilized membranes from CHO cells expressing human SPP.

into E. coli membranes (Fig. 1a). Expression of these fusion proteins was confirmed by Western blot of total cell lysates, with some degree of protein degradation also being observed (not shown). E. coli membranes were isolated and solubilized in 1% DDM. This non-ionic detergent with a low critical micelle concentration was identified as best for protein extraction and provided less interference with protease activity among eight detergents tested (data not shown).

Each of the expressed MBP-SPP fusion proteins were tested for proteolytic activity using an established in vitro assay that utilizes a synthetic Myc and FLAG-tagged peptide based on the prolactin signal sequence (Prl-FLAG) (15). Cleavage products were identified by Western blot analysis after incubating solubilized membranes with substrate at 30 °C for 90 min (Fig. 1b). Proteolytic fragments from the Prl-FLAG substrate were not observed before incubation and were prevented by a specific SPP inhibitor, (ZLL)\textsubscript{2} ketone. Moreover, proteolysis was observed only in the presence of isopropyl 1-thio-β-d-galactopyranoside induction (data not shown), and solubilized membranes from E. coli expressing MBP alone could not cleave the substrate (Fig. 1b), indicating that the proteolysis was not due to endogenous E. coli proteins. Site-directed mutagenesis of either of the two conserved transmembrane aspartates eliminated the SPP activity (Fig. 1c), consistent with the hypothesis that these residues represent the active site of a membrane-embedded aspartyl protease. Moreover, the sensitivity of this protease activity to a panel of inhibitors was consistent with that expected for SPP activity (13, 15); (ZLL)\textsubscript{2} ketone reduced the proteolytic activity, but none of the other more broad-spectrum inhibitors (pepsstatin A, dichloroisocoumarin (DCI), E-64, and bestatin) significantly affected activity (Fig. 1d). Human and Drosophila SPP displayed pH optima closer to neutral pH, whereas S. pombe SPP showed a pH optimum at acidic pH (data not shown). Together these results suggest that these expressed MBP-SPPs from diverse organisms are functional.

Expression and proteolytic activity were similarly observed when Drosophila SPP was transformed with a C-terminal His\textsubscript{6} tag instead of an N-terminal MBP (Fig. 1, e and f). The observed Prl-FLAG cleavage product co-migrated on the gel with product formed using membranes from Chinese hamster ovary (CHO) cells stably expressing human SPP. Time-dependent product formation and concentration-dependent inhibition with (ZLL)\textsubscript{2} ketone were also observed (data not shown). These results clearly demonstrate that proteolytic activity was due to the expressed SPP protein, and this activity closely resembles that seen in mammalian cells.

Reconstitution of SPP Activity with Purified Protein—Biochemical and structural understanding of SPP demands purification and functional reconstitution of this enzyme in lipids. DDM was chosen as the detergent to isolate SPP in functional form. Previous studies showed that over time DDM completely disrupted interaction of presenilin with its co-factors in the γ-secretase complex and abolished activity (19). Any E. coli proteins that interact with SPP might be expected to dissociate during the purification process in the presence of DDM, allowing purification of the protein and testing of the hypothesis that SPP functions as a protease on its own. Drosophila MBP-SPP was used for this study because it showed better proteolytic activity and appeared to degrade less than the other SPPs after optimization of expression. After membrane isolation, affinity purification was carried out on amylose beads with maltose elution, providing pure protein as assessed by SDS-PAGE with Coomassie Blue or silver staining (Fig. 2, a–c). Previously, SPP was shown to exist as an SDS stable dimer that could be labeled with a transition-state analogue affinity probe (14). Based on these and other findings, dimerization was thought to be important for activity (14, 20). However, although SDS-stable dimers could be observed after DDM solubilization of membranes (Fig. 2a), none were seen after affinity purification (Fig. 2b).

The activity of this pure protein was reconstituted with lipids, which play crucial roles in the function of enzymes located within the membrane (21). Lipids were chosen to provide variation in the polar head groups. E. coli total lipids, primarily composed of phosphatidylethanolamine (PE) and phosphatidylglycerol, were selected first because SPP activity was seen in E. coli membrane extracts. Other lipids tested included 1) phosphatidylcholine (PC), 2) a 3:1 PC/PE mixture, and 3) heart total lipids, a combination of the zwitterionic PC and PE and the anionic phosphatidylinositol and phosphatic acid. Robust activity was observed in the presence of PC or PE/PC and was highest with E. coli total lipids; cardiac total lipids provided virtually no activity (Fig. 2d). Proteolytic activity required the added lipids and was completely inhibited by (ZLL)\textsubscript{2} ketone. γ-Secretase and Rhomboid both likewise required added lipids after affinity purification for proteolytic activity (19, 22, 23), suggesting that this is a general property of 1-CLiPs. Presumably, specific lipids and detergents allow these proteases to
assume conformations that are conducive to proper interaction with and handling of substrate and/or that allow appropriate geometric alignment between catalytic residues.

Identification of a Four-TM Functional Domain of SPP—The *in vitro* proteolytic SPP assay showed that human, *S. pombe*, and *Drosophila* SPP as well as *Drosophila* SPP-like protein 3 could all cleave the same synthetic substrate based on the bovine prolactin signal sequence. In each case the activity was blocked by the specific SPP transition-state analogue inhibitor, (ZLL)$_2$ ketone, and the cleaved product formed from the bacterially expressed SPP co-migrated with product formed from solubilized membranes from mammalian cells (Fig. 1, b and f). Although the functional implications of these observations *in vivo* are not clear, the biochemical nature of the active site and substrate binding site are apparently highly conserved throughout these organisms. Interestingly, sequence alignment of the SPPs from these organisms showed higher homology in the C-terminal region compared with the N-terminal region (supplemental Fig. 1). The sequences containing the two essential aspartates were highly conserved as is a signature PAL domain further downstream.

This observation led to the hypothesis that the C-terminal portion of SPP may represent a functional domain for enzymatic activity and that this domain may be capable of carrying out proteolysis on its own. This idea, however, had potential problems. Residues not involved in catalysis and located in disparate regions of the protein may nevertheless serve to stabilize the functional domain. For presenilin, evidence suggests that TM1 interacts with TM8 and TM3 with TM6 (24–27), with the prediction that removing TM1 or TM3 would impair enzymatic activity. Evidence suggests that the membrane topology of SPP is opposite that of presenilin, with either seven or nine TM domains (17, 18), and our computational analysis suggested that SPP contains at least six TM domains. In any event, truncation of SPP just before the TM domain containing the first essential aspartate is predicted to provide a protein with four TM domains altogether. Loop regions were chosen to define the boundary of the functional domain. Combining our biochemical data, sequence homology, and topology analysis, the functional domain was defined as represented by the two arrows in the sequence alignment (supplemental Fig. 1) and by arrowheads in the schematic representation (Fig. 1a).

The so-defined functional domains from human, *S. pombe*, and *Drosophila* SPP were subcloned into a vector that allowed installation of the PelB leader sequence on the N terminus and a His$_6$ tag on the C terminus. After expression, the membranes were isolated, and all three functional domains were found to be inserted into the membrane, presumably due to the presence of the pelB signal sequence in the plasmid (Fig. 3a). Degradation bands were not observed; however, because the His$_6$ epitope tag for antibody detection and purification was placed at the C terminus, premature translation and degradation cannot be ruled out. After solubilization of membranes in 1% DDM, the SPP activity assay was performed, and the three functional domains from each species were all proteolytically active (Fig. 3b). The degree of activity in each case was comparable with that seen using full-length *Drosophila* SPP, and the cleaved products co-migrated with this positive control. These results

![Figure 2. Purification of *Drosophila* MBP-SPP and reconstitution of activity. a, Western blot using anti-MBP antibodies and Coomassie (b) and silver staining analyses (c) of the MBP-SPP fusion protein after affinity purification from amylose beads showed a single band with the expected mobility. d, activity of MBP-SPP fusion protein reconstituted using various lipids. Activity was specifically inhibited by (ZLL)$_2$ ketone.](image)

![Figure 3. Expression in *E. coli* of the SPP functional domain from several organisms provides active protease. a, human (*HU*), *S. pombe* (*Pom*), and *Drosophila* (*Dro*) SPP functional domains (FD) expressed in *E. coli* membranes as probed by anti-penta-His antibody. b, DDM-solubilized functional domains showed similar proteolytic activity compared with WT full-length *Drosophila* SPP. Proteolytic activity was specifically inhibited by (ZLL)$_2$ ketone, 0, 90, and 90 min with (ZLL)$_2$ ketone, respectively. DDM-solubilized membranes expressing vector alone showed no activity. c, expression of MBP-fused human SPP functional domain as probed by anti-penta-His antibody. d, proteolytic activity of DDM-solubilized human SPP functional domain and its inhibition by (ZLL)$_2$ ketone. e, mutating either conserved aspartate to alanine drastically reduced activity of the functional domain. f, inhibitors of different classes of proteases showed no effect on human SPP functional domain activity, whereas (ZLL)$_2$ ketone reduced activity significantly. DCI, 3,4-dichloroisocoumarin.](image)
Intramembrane Aspartic Protease Functional Domain

imply that activity was not compromised in the engineered functional domain of SPP.

Because affinity purification using amylose beads provided highly pure samples of full-length SPP, an N-terminal MBP fusion was introduced into the functional domain as well. Expressed MBP-fused human SPP was detected in DDM-solubilized E. coli membranes as a single band by anti-MBP Western blot (Fig. 3c). These solubilized membranes contained SPP-like proteolytic activity, whereas membranes containing MBP alone did not (Fig. 3d). Mutagenesis showed that changing either one of the essential and conserved aspartates abolished the proteolytic activity of the functional domain (Fig. 3e). Moreover, the MBP-fused functional domain displayed the same profile of inhibitor sensitivity as full-length SPP; only the specific SPP inhibitor (ZLL)$_2$ ketone substantially reduced activity (Fig. 3f), although the extent of this inhibition was not as great as that seen with full-length SPP. The inhibition by (ZLL)$_2$ ketone was concentration-dependent, with an IC$_{50}$ of $\sim 15$ µM compared with 2 µM full-length SPP (not shown). Taken together, these findings suggest that the chemistry of proteolysis is the same for both the full-length protein and the functional domain and that the four C-terminal transmembrane domains may be sufficient for SPP proteolytic activity.

Reconstitution of SPP Activity with Purified Functional Domain—To corroborate the concept of the functional domain, human, S. pombe, and Drosophila SPP functional domains were purified by affinity column. DDM-solubilized membranes containing MBP-fused SPP were loaded onto amylose beads, washed, and eluted with maltose. MBP-SPP fractions contained a significant amount of MBP alone, cleaved from the SPP portion (not shown). Reconstitution of proteolytic activity was attempted with PC as well as E. coli total lipids. Whereas both types of lipids could reconstitute activity with purified full-length Drosophila (Fig. 2d), only E. coli total lipids worked for the functional domain (Fig. 4a). The reason for this difference is not clear; nevertheless, these findings suggest that the functional domain is proteolytically active without the need for any other protein factors (the free MBP in these samples does not have proteolytic activity; see Fig. 3d). SPP functional domains from all three species were also reconstituted successfully in this way (data not shown).

Removal of the MBP fusion protein would provide a functional domain of $\sim 15$ kDa, putting this membrane protein within range suitable for study by NMR. Two lipid-mimicking detergents that are suitable for NMR, foscholine-12 and 1,2-dimyristoyl-sn-glycero-3-phosphocholine, were tested for compatibility with protease activity, swapping out the DDM during the affinity purification step. Including 2% glucose in the E. coli media and reducing the post-induction temperature to 20°C together eliminated degradation of MBP-fused human SPP functional domain and facilitated high level isolation (Fig. 4b). Human SPP functional domain was purified to virtual homogeneity as assessed by Coomassie and silver staining (Fig. 4, c and d). Proteolytic activity was not seen in 1,2-dimyristoyl-sn-glycero-3-phosphocholine or DDM; however, foscholine-12 detergent was compatible with activity in the absence any added lipids (Fig. 4f). Glycerol velocity gradient experiments were performed to understand the distribution of native functional SPP in foscholine-12 detergent. The results suggested that both functional domain and wild type full-length SPP fractioned as monomers, indicating monomers as active component of SPP (monomer for human FD-SPP and SPP is calculated as 65 and 88 kDa, respectively) (Fig. 4e). We also purified the human SPP functional domain containing only the C-terminal His$_6$ tag using a nickel column and eluting with imidazole (Fig. 4g). This purified material ran as a monomeric 15-kDa protein on native PAGE, with no sign of dimerization or higher order aggregates (Fig. 4h). Reconstitution of proteolytic activity was seen using E. coli total lipids (Fig. 4i), suggesting that the functional domain is active as a monomer and demonstrating conclusively that this four transmembrane C-terminal region is sufficient for SPP activity without requiring additional protein factors.

The Functional Domain of SPP Is Sensitive to Inhibitors Directed to the Active Site or to the Docking Site—The hydrolytic machinery for intramembrane proteolysis has been proposed to be buried inside the polytopic protease, sequestered...
Intramembrane Aspartic Protease Functional Domain

from the hydrophobic environment of the lipid bilayer, a hypothesis confirmed by the recent crystal structures of Rhomboids (2–4). Helical transmembrane substrates are proposed to interact initially with a hydrophobic region on the lipid-exposed surface of the enzyme (the docking site) before lateral gating allows movement in whole or in part into the internal active site (28). Helical peptide substrate mimics are potent inhibitors of γ-secretase and bind directly to presenilin at a location distinct from the active site and consistent with a substrate docking site (25). Evidence using these helical peptide probes further suggests that the active site and docking site are quite close. A substrate-based helical peptide designed from the prolactin signal sequence can also effectively inhibit SPP, and affinity labeling experiments suggest that this helical peptide binding site is distinct from the active site (15). A set of additional helical inhibitors has been recently designed on the basis of another SPP substrate, the calreticulin signal sequence (3). Both full-length SPP and the functional domain appear to be closely similar.

In the same way, a set of peptidomimetics containing a transition-state-mimicking hydroxyl moiety was tested for effects on the full-length SPP vis-à-vis the functional domain. These compounds are directed to the active site of aspartyl proteases and can serve as probes for the topography of the enzyme near the catalytic aspartates. Similar studies have been carried out on the presenilin-containing γ-secretase complex (16). We observed some similarities between full-length SPP and the functional domain in the inhibition profile of these transition-state analogue inhibitors (Fig. 5b). For instance, both were effectively inhibited by the all-phenylalanine-containing analogue 12, and neither was at all affected by analogue 9. However, important differences were noted as well. Inhibitors closely related to 12 (analogues 11 and 13) still inhibited full-length SPP to some extent but had no effect on the functional domain. In contrast, analogue 10 could inhibit the functional domain to some extent but had no effect on the full-length protease, and analogue 8 was very effective against the functional domain compared with the full-length SPP. Nevertheless, these results altogether suggest that the functional domain contains similar binding sites for these two different types of inhibitors and that this engineered, truncated protease contains similar docking and active sites.

**DISCUSSION**

I-CLiPs carry out hydrolysis within the confines of the lipid bilayer and play many crucial roles in biology and disease. The workings of these novel membrane-embedded enzymes have largely been elucidated through molecular and cellular approaches, but the focus has recently turned to biochemical purification and structural studies to gain more detailed understanding. In this regard, the successful expression and purification of serine I-CLiPs, the Rhomboid family, have led to several new crystal structures that together confirm the nature of the catalytic site and provide new mechanistic insights. Although the elucidation of these new structures is a tour de force in the study of I-CLiPs, it is unclear which principles of Rhomboid structure and catalysis can be generalized to other I-CLiPs. Only the structural elucidation of members of other I-CLiP families will reveal general principles.

Aspartyl I-CLiPs may share some general features with Rhomboids, but there are no doubt many critical differences as well. Indeed, two low resolution electron microscopy structures of aspartyl I-CLiPs, both of the γ-secretase complex isolated from eukaryotic cells, reveal the existence of a large central chamber (9, 10). This is in sharp contrast to the horseshoe shape of rhomboids, which putatively allows water and substrate accessibility (2–4). Despite this advance, the detailed proteolytic mechanism of γ-secretase will only be explained by atomic resolution structures, a tough challenge for this complex containing presenilin and three other membrane proteins. However, SPP is also an aspartyl I-CLiP, and evidence suggested that SPP does not require other protein factors. Hence, SPP may be considered a prototype to understand aspartyl intramembrane proteolysis.
Intramembrane Aspartic Protease Functional Domain

Understanding the molecular mechanism of the SPP family of proteases requires functional reconstitution of pure components in vitro. This is considered as a first and foremost step for any structural and biochemical characterization. To date, three out of four I-CLiP families have been successfully reconstituted (22, 23, 29, 30). We have shown here that SPP from divergent organisms can be expressed in E. coli and are proteolytically active as purified proteins. This finding formally demonstrates that SPP alone is a protease. Like γ-secretase and Rhomboid, SPP requires added lipids to reconstitute proteolytic activity after purification, and this feature, thus, appears to be a general characteristic of membrane-embedded proteases (22, 23, 30).

The activity of the purified and reconstituted SPP mimics that of SPP isolated from mammalian cells in terms of both the size of the proteolytic product generated and the susceptibility to a transition-state analogue inhibitors, with the implication that the functional domain still possesses a substrate docking site as well as the active site. In one of the recent reports on the structure of the E. coli Rhomboid GIpG, the C terminus was similarly proposed as a functional domain containing the active site Ser-Her dyad as well as a site for substrate access through a lateral gating mechanism (2, 4). However, the N terminus appears to serve an essential role in supporting this functional domain and is postulated to be a structural scaffold (2). Also, biochemical studies of presenilin indicate the N terminus is crucial for activity (24). Nevertheless, our findings indicate that a conserved C-terminal region of SPP is sufficient for the proteolytic activity and suggests a novel structural feature of SPP compared with other I-CLiPs.

In light of our findings, the biochemical function of the SPP N terminus is unclear. SPP appears to play at least one other non-proteolytic role, helping to extrude misfolding membrane proteins as part of a quality control mechanism in the endoplasmic reticulum (32). Similarly, presenilin apparently has functions independent of its role in the γ-secretase complex, serving as a component of a clock critical to somite formation during development and also as a putative calcium channel in the endoplasmic reticulum (33, 34). The N-terminal regions of SPP and presenilin may be critical for the evolution of these non-proteolytic functions.

Both functional domain and full-length SPP are fractionated as monomers in glycerol velocity gradient. Also, the active SPP functional domain runs as a monomer on native gel, evidence that SDS-stable dimerization is not required for proteolytic activity. Thus, although both SPP and presenilin have been postulated to function as SDS-stable homodimers (14, 35), our findings suggest that certain models for the active site (i.e. those containing two pairs of aspartates) can be reasonably excluded. However, transient dimer formation in the presence of lipids or substrates cannot be ruled out. The small size of the functional domain, only ~15 kDa, suggests that this protein may be amenable to structural studies by NMR. The remarkable finding that this small, engineered SPP possesses activity closely resembling that of the full-length protein suggests that this functional domain is a prototype to understand the structure and proteolytic mechanism of the aspartyl I-CLiP family and a convenient model for discerning general biochemical principles that underlie intramembrane proteolysis.

Acknowledgments—We thank A. Kuppanna for the helical peptides and S. Urban and P. Fraering for helpful discussions.

REFERENCES
1. Wolfe, M. S., and Kopan, R. (2004) Science 305, 1119–1123
2. Wu, Z., Yan, N., Feng, L., Oberstein, A., Yan, H., Baker, R. P., Gu, L., Jeffrey, P. D., Urban, S., and Shi, Y. (2006) Nat. Struct. Mol. Biol. 13, 1084–1091
3. Ben-Shem, A., Fass, D., and Bibi, E. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 462–466
4. Wang, Y., Zhang, Y., and Ha, Y. (2006) Nature 444, 179–180
5. Selkoe, D. J., and Kopan, R. (2003) Annu. Rev. Neurosci. 26, 565–597
6. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Nat. Cell Biol. 5, 486–488
7. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6382–6387
8. Takasuji, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niihara, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) Nature 422, 438–441
9. Lazarov, V. K., Fraering, P. C., Ye, W., Wolfe, M. S., Selkoe, D. J., and Li, H. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6889–6894
10. Ogura, T., Mio, K., Hayashi, I., Miyashita, H., Fukuda, R., Kopan, R., Kodama, T., Hamakubo, T., Iwatsubo, T., Tomita, T., and Sato, C. (2006) Biochem. Biophys. Res. Commun. 343, 525–534
11. Martoglio, B., and Golde, T. E. (2003) Hum. Mol. Genet. 12, 201–206
12. Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K., and Martoglio, B. (2002) Science 296, 2215–2218
13. Weihofen, A., Lemberg, M. K., Friedman, E., Rueeger, H., Schmitz, A., Paganetti, P., Rovelli, G., and Martoglio, B. (2003) J. Biol. Chem. 278, 16528–16533
14. Nyborg, A. C., Kornilova, A. Y., Jansen, K., Ladd, T. B., Wolfe, M. S., and Golde, T. E. (2004) J. Biol. Chem. 279, 15153–15160
15. Sato, T., Nyborg, A. C., Jwata, N., Diehl, T. S., Saido, T. C., Golde, T. E., and Wolfe, M. S. (2006) Biochemistry 45, 8649–8656
16. Eser, W. P., Das, C., and Wolfe, M. S. (2004) Bioorg. Med. Chem. Lett. 14, 1935–1938
17. Friedmann, E., Lemberg, M. K., Weihofen, A., Dev, K. K., Dengler, U., Rovelli, G., and Martoglio, B. (2004) J. Biol. Chem. 279, 50790–50798
18. Nyborg, A. C., Jansen, K., Ladd, T. B., Fauq, A., and Golde, T. E. (2004) J. Biol. Chem. 279, 43148–43156
19. Fraering, P. C., LaVoie, M. J., Ye, W., Ostaszewski, B. L., Kimberly, W. T., Selkoe, D. J., and Wolfe, M. S. (2004) Biochemistry 43, 323–333
20. Wang, J., Beer, D., Nyborg, A. C., Shearman, M. S., Golde, T. E., and Goate, A. (2006) J. Neurochem. 96, 218–227
21. Lee, A. G. (2004) Biochim. Biophys. Acta., Biomembr. 1666, 62–87
22. Lemberg, M. K., Menendez, J., Misik, A., Garcia, M., Koth, C. M., and Freeman, M. (2005) EMBO J. **24**, 464–472
23. Urban, S., and Wolfe, M. S. (2005) Proc. Natl. Acad. Sci. U. S. A. **102**, 1883–1888
24. Brunkan, A. L., Martinez, M., Wang, J., Walker, E. S., Beher, D., Shearman, M. S., and Goate, A. M. (2005) J. Neurochem. **94**, 1315–1328
25. Kornilova, A. Y., Bihel, F., Das, C., and Wolfe, M. S. (2005) Proc. Natl. Acad. Sci. U. S. A. **102**, 3230–3235
26. Kornilova, A., Kim, J., Laudon, H., and Wolfe, M. S. (2006) Biochemistry **45**, 7598–7604
27. Annaert, W. G., Esselens, C., Baert, V., Boeve, C., Snellings, G., Cupers, P., Craessaerts, K., and De Strooper, B. (2001) Neuron **32**, 579–589
28. Esler, W. P., Kimberly, W. T., Ostaszewski, B. L., Ye, W., Diehl, T. S., Selkoe, D. J., and Wolfe, M. S. (2002) Proc. Natl. Acad. Sci. U. S. A. **99**, 2720–2725
29. Akiyama, Y., Kanehara, K., and Ito, K. (2004) EMBO J. **23**, 4434–4442
30. Fraering, P. C., Ye, W., Strub, J. M., Dolios, G., LaVoie, M. J., Ostaszewski, B. L., van Dorsselaer, A., Wang, R., Selkoe, D. J., and Wolfe, M. S. (2004) Biochemistry **43**, 9774–9789
31. Ponting, C. P., Hutton, M., Nyborg, A., Baker, M., Jansen, K., and Golde, T. E. (2002) Hum. Mol. Genet. **11**, 1037–1044
32. Loureiro, J., Lilley, B. N., Spooner, E., Noriega, V., Tortorella, D., and Ploegh, H. L. (2006) Nature **441**, 894–897
33. Huppert, S. S., Ilagan, M. X., De Strooper, B., and Kopan, R. (2005) Dev. Cell **8**, 677–688
34. Tu, H., Nelson, O., Bezprozvanny, A., Wang, Z., Lee, S. F., Hao, Y. H., Serneels, L., De Strooper, B., Yu, G., and Bezprozvanny, I. (2006) Cell **126**, 981–993
35. Schroeter, E. H., Ilagan, M. X., Brunkan, A. L., Hecimovic, S., Li, Y. M., Xu, M., Lewis, H. D., Saxena, M. T., De Strooper, B., Coonrod, A., Tomita, T., Iwatsubo, T., Moore, C. L., Goate, A., Wolfe, M. S., Shearman, M., and Kopan, R. (2003) Proc. Natl. Acad. Sci. U. S. A. **100**, 13075–13080