Three-dimensional Structure of the Signal Peptide Peptidase

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Signal peptide peptidase (SPP) is an atypical aspartic proteinase that hydrolyzes peptide bonds within the transmembrane domain of substrates and is implicated in several biological and pathological functions. Here, we analyzed the structure of human SPP by electron microscopy and reconstructed the three-dimensional structure at a resolution of 22 Å. Enzymatically active SPP forms a slender, bullet-shaped homotetramer with dimensions of 85 × 85 × 130 Å. The SPP complex has four concaves on the rhombus-like sides, connected to a large chamber inside the molecule. Intriguingly, the N-terminal region of SPP is sufficient for the tetrameric assembly. Moreover, overexpression of the N-terminal region inhibited the formation of the endogenous SPP tetramer and the proteolytic activity within cells. These data suggest that the homotetramer is the functional unit of SPP and that its N-terminal region, which works as the structural scaffold, has a novel modulatory function for the intramembrane-cleaving activity of SPP.

The intramembrane-cleaving proteases (I-CLiPs) that sever the transmembrane domains of their substrates have been identified in a range of organisms and play a variety of roles in biological and pathological conditions (1). I-CLiPs have been classified into three groups: serine-, aspartyl-, and metalloproteinase-type, according to the structure of active sites. Presenilin (PS) and signal peptide peptidase (SPP) family proteins belong to the group of aspartyl I-CLiPs (2, 3). These polytopic proteinases have nine transmembrane domains with the two catalytic aspartates as YD and GDDS motifs. Several γ-secretase inhibitors cross-inhibit the SPP activity, suggesting that PS, the catalytic subunit of γ-secretase, and SPP share a similar structure and proteolytic mechanism (4–8). However, γ-secretase requires three cofactor proteins (i.e. nicastrin, aph-1, and pen-2) in addition to PS (9–11), whereas SPP alone exhibits catalytic function not requiring other protein cofactors (4). SPP is implicated in the clearance of signal peptides as well as misfolded membrane proteins (12–14). Moreover, some endoproteolytic products generated by SPP cleavage directly mediate signal transduction (15, 16). In fact, loss-of-function studies of SPP in model animals resulted in severe developmental defects, inferring a vital role of SPP in metazoan development (17–19). Furthermore, a growing body of evidence indicates that SPP activity plays an important role in the maturation of several pathogens including the hepatitis C virus and the malaria parasite (7, 20). Thus, understanding the structure and function relationship of SPP as well as the rational development of its inhibitors should have a significant therapeutic potential for these infectious diseases. Here, we found that SPP proteins formed a tetramer in the enzymatically active condition. Single particle reconstruction from electron microscopic images revealed that the purified SPP forms a bullet-like shape with concaves on the surface and a large chamber in the center. Intriguingly, overexpression of the N-terminal region of SPP, which is sufficient for the tetrameric assembly, led to the inhibition of the proteolytic activity. Our first study on the structure of SPP reveals its submolecular configuration and highlights a novel modulatory mechanism of the N-terminal region on the proteolytic activity of SPP.

EXPERIMENTAL PROCEDURES

Antibodies and Compounds—Rabbit polyclonal antibodies dSPP-N1 and dSPP-C1 were raised against glutathione S-transferase fused to amino acids 1–20 and 370–389 of Drosophila SPP (dSPP), respectively. Rabbit polyclonal antibody anti-
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GDN1 against N-terminal region of *Drosophila* presenilin (Psn) was previously described (21). Rabbit polyclonal antibody SPP<sub>CT</sub> against the C terminus (358–377) of human SPP was kindly provided by Dr. Todd Golde (University of Florida) (22). Mouse monoclonal antibodies were purchased from Sigma (anti-FLAG M2), Invitrogen (anti-V5), Qiagen (Hilden, Germany) (anti-H<sub>5</sub>), and Cell Signaling Technology (Danvers, MA) (anti-c-Myc 9B11), respectively. ([2R,4R,5S]-2-Benzyl-5-(t-butyloxycarbonylamino)-4-hydroxy-6-phenylhexanoyl]-l-leucyl-l-phenylalanine amide (L-685,458) (23) and 1,3-di-(N-carboxybenzoyl-l-leucyl-l-leucyl)amino acetone ((Z-LL)<sub>2</sub>-ketone) (12) were purchased from the PEPTIDE INSTITUTE INC. (Osaka, Japan). pep.11 and pep.11-Bt were synthesized by BEX Co., LTD (Tokyo, Japan). L-852,505, L-852,646, Dibenzazepine (DBZ), DBZ-BpB3, 31C, and 31C-Bpa were kindly provided by Drs. Yueming Li (Memorial Sloan-Kettering Cancer Center, NY), Haruhiko Fuwa (Tohoku University, Miyagi, Japan) Naoki Umezawa and Tsunehiko Higuchi (Nagoya City University, Aichi, Japan) (23–25).

**Construction of Expression DNA Plasmids, Cell Culture, Recombinant Baculovirus Generation, and Transfection—**Full-length cDNAs encoding wild-type (WT) human SPP as well as D219A mutant SPP (kindly provided by Dr. Evgeny I. Rogaev (University of Massachusetts Medical School) (17)) were inserted into pCMV10 (Sigma) vector to add 3×FLAG tag at the N terminus. 3×FLAG-tagged human SPP (3SFSP) cDNAs were then inserted into pFASTBac 1 vector (Invitrogen). cDNA encoding 3SFSP/NT in pFASTBac 1 vector was synthesized by PCR. cDNAs encoding full-length dSPP and dSPP/NT were amplified using I.M.A.G.E. clone SD07518 and inserted into pAc5.1/V5-His B vector (Invitrogen). Aspartate mutant dSPP (i.e. D228A or D274A) as well as dSPP/NT was generated by PCR. cDNAs encoding SPP<sub>sub</sub> (kindly provided by Drs. Andrew Nyborg and Todd Golde) (22) as well as *Renilla* luciferase (Promega) were subcloned into pB/V5-His/TOPO vector (Invitrogen). Endoplasmic reticulum stress response element (ERSE)-fused firefly luciferase construct (kindly provided by Dr. Kazutoshi Mori (Kyoto University, Kyoto, Japan) (26)) was inserted into pIZT/V5-His vector (Invitrogen). Expression construct for SPP.L2b in pEF4/myc-His (Invitrogen) was provided by Drs. Regina Fluhler and Christian Haass (Ludwig-Maximilians-University, München, Germany) (19). All constructs were sequenced using Thermo Sequenase (GE Healthcare) on an automated sequencer (LI-COR Biosciences, Lincoln, NE). Recombinant baculovirus was generated according to the manufacturer’s instructions. Maintenance and transfection of S2 and S9 cells were performed as described previously (11, 21, 27, 28). S9 cells (2 × 10<sup>6</sup> cells/ml) were infected with a combination of recombinant baculoviruses at the virus volume of maximum expression level and harvested 72 h after infection.

**Sample Preparation, Immunoprecipitation, Photoaffinity Labeling, Electrophoresis, and Immunoblotting—**The cells were homogenized in 10% w/v glycerol-containing HEPEs buffer (10 mM HEPEs, pH 7.4, 150 mM NaCl, and Complete protease inhibitor mixture (Roche Applied Science)) and subsequently centrifuged at 1,000 × g for 10 min. The supernatants were centrifuged again at 100,000 × g for 60 min to isolate the microsome fraction. The microsomes or cells were resuspended in 2% n-dodecyl-β-D-maltopyranoside (DDM)-containing HEPES buffer to designate them as the solubilized microsomes or cell lysates. For large scale preparation, solubilized microsome fractions from infected S9 cells were loaded on anti-FLAG M2-agarose and eluted with 500 μg/ml 3×FLAG peptide-containing 2% DDM-containing HEPES buffer after being washed three times. Eluate was further separated by a Superose 6 HR 10/30 column (GE Healthcare) on an AKTA Explorer chromatography system (GE Healthcare). For immunoprecipitation, DDM-solubilized fractions were coincubated with primary antibody and protein G-agarose (Invitrogen). Photoaffinity labeling experiments were performed as described previously, with some modifications (24, 25, 29). Briefly, microsomal fractions or 0.25% DDM-solubilized fractions were incubated with 50 nm photoprobes (i.e. L-852,505, L-852,646) and then irradiated for 90 min. Irradiated samples were adjusted to 1% SDS and rocked with immobilized streptavidin (GE Healthcare) overnight. Biotinylated proteins were eluted with SDS sample buffer by heating for 1 min and subjected to immunoblotting. SDS-PAGE and immunoblotting were performed as described previously (30). Blue-Native PAGE (BN-PAGE) was performed according to the manufacturer’s protocol (Invitrogen). Briefly, membrane fractions were suspended in NativePAGE<sup>TM</sup> sample buffer containing 1% DDM. The mixture was centrifuged for 10 min at 15,000 × g, and Coomassie Brilliant Blue was added to the supernatant to give a final concentration of 0.25% w/v. NativeMark<sup>TM</sup> unstained protein standard (Invitrogen) was used as a molecular weight standard. After electrophoresis, the gel was transferred to PVDF membranes. The membranes were destained briefly in methanol before being incubated with specific antibodies.

**SPP Activity Assay—**To measure SPP activity in vitro, solubilized samples were incubated with Myc-Prl-PP-FLAG peptide (BEX Co., Ltd.) (31) at 37 °C. For SPP reporter assay in vivo (22), constructs encoding dSPP, ERSE-firefly luciferase, SPP<sub>sub</sub>, and *Renilla* luciferase were transfected into S2 cells. Luciferase activities were measured by the PicaGene Dual luciferase system (TOYO B-Net. Co., LTD. Tokyo, Japan) according to the manufacturer’s instructions.

**Transmission Electron Microscopy—**Fragments were adsorbed by thin carbon films rendered hydrophilic by glow discharge in low air pressure and supported by copper mesh grids. Samples were washed with five drops of double-distilled water, negatively stained with 2% uranyl acetate solution for 30 s twice, blotted, and air-dried. For immuno-EM, purified 3SFSP and anti-FLAG M2 monoclonal antibody were mixed for 90 min at 4 °C, and excess antibodies were removed by gel filtration chromatography (SMART system (GE Healthcare)). Samples were then negatively stained as described. Micrographs of negatively stained particles were recorded in a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan) at ×53,100 magnification with 100-kV acceleration voltages. Images were recorded on SO-163 films (Eastman Kodak Co.), developed with a D19 developer (Kodak) and digitized with a Scitex Leafscan 45 scanner (Leaf systems, Westborough, MA) at a pixel size of 1.92 Å at the specimen level.

**Automated Particle Selection and Image Analysis—**Single particle image analysis (32) was performed using our SPINNS
program and the IMAGIC V program (33–37). The projections were picked up by a combination of two automatic pickup programs: the auto-accumulation method using simulated annealing (34) and the three-layered neural network method (35). Initially, 531 particles, in 160 × 160-pixel subframes, were selected from five EM images and used to train a pyramid-type neural network. Using the trained neural network, 4,692 particles were selected. The images were band pass-filtered with a low frequency cutoff of 384 Å and a high frequency cutoff of 4 Å, using IMAGIC V. The following image analysis was performed in three steps. First, the 4,692 images were rotationally and translationally aligned using the reference-free method (35). The aligned images were then classified into 150 clusters by the modified growing neural gas network method using a circular mask (37). Images in each cluster were averaged, and the averages with circular mask were used as new references. This cycle, from alignment to averaging, was repeated 25 times. The Euler angles of the class averages were automatically determined by the echo-correlated three-dimensional reconstruction method with simulated annealing (36) assuming C4 symmetry because of the tetrameric subunit stoichiometry of 3FSPP. These angles were used to calculate a preliminary three-dimensional density map by the simultaneous iterative reconstruction technique (38). The reprojections from the volume were employed as references for multi-reference alignment, and each image in the library was aligned and classified, providing improved cluster averages. From these averages, a new three-dimensional map was generated by the reconstruction method using simulated annealing without a three-dimensional reference. This cycle was repeated for three cycles. The density map was further refined by projection matching (39) followed by echo-correlated reconstruction. This cycle was repeated until convergence. Resolution was assessed without masking by dividing the data into two subsets and then calculated using the independent three-dimensional reconstructions of each, which were compared by Fourier shell correlation at the threshold of 0.5, using IMAGIC V.

RESULTS

Proteolytically Active SPP Polypeptides Form a Multimeric Complex—To characterize the SPP molecule in an enzymatically active state, we analyzed endogenous SPP by BN-PAGE in the DDM-solubilized condition, in which SPP activity was preserved (31). Endogenous human SPP, which existed as a 45-kDa monomer and a heat-sensitive dimer (90 kDa) on SDS-PAGE analysis (40) (Fig. 1A), was detected as a single band at 200 kDa (Fig. 1B), suggesting that active SPP forms a high molecular mass complex in the DDM-solubilized condition. Next we analyzed Drosophila SPP, which is composed of 389 amino acids (18). Endogenous dSPP polypeptide in S2 cells was detected as a single band of 40 kDa (Fig. 1C) without extensive boiling, indicating that dSPP did not form the SDS-resistant dimer (Fig. 1D). However, DDM-solubilized dSPP was migrated as a 180-kDa high molecular mass complex (Fig. 1D). Notably, endogenous dSPP was specifically co-immunoprecipitated with exogenously overexpressed dSPP, but not with other multispanning proteins, similarly to mammalian SPP (Fig. 1E, supplemental Fig. S1A) (22, 40), suggesting that the ability of SPP to form a

FIGURE 1. Endogenous SPP proteins formed a high molecular weight complex in the enzymatically active state. A, immunoblot analysis of SDS-solubilized HEK293 cell lysates. Samples were loaded with (+) or without (−) pre-boiling (Boil). The SDS-resistant dimer and monomer forms of SPP are indicated by the black arrowhead and arrow, respectively. B, SPP proteins (black arrow) in 0.25% DDM-solubilized HEK293 cell lysates were analyzed by BN-PAGE. C, immunoblot analysis of SDS-solubilized lysates of S2 cells transfected with dsRNA (RNAi), mock, or dSPP-V5. Endogenous and overexpressed dSPP proteins are indicated by the black arrow and white arrowhead, respectively. D, dSPP proteins (black arrow) in 0.25% DDM-solubilized HEK293 cell lysates were analyzed by BN-PAGE. E, immunoprecipitation of DDM-solubilized S2 cells transfected with mock or dSPP-V5 by antibodies indicated at the top of the panels. 10% of lysates was loaded as an input control. Endogenous and overexpressed dSPP proteins are indicated by the black arrows and white arrowheads, respectively. iPd, immunoprecipitated.
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homooligomer is conserved beyond species irrespective of the formation of SDS-resistant dimer. In addition, DDM-solubilized SPP L2b, of which the mature form migrated at 90 kDa on SDS-PAGE (19), was detected at 400 kDa on BN-PAGE (supplemental Fig. S1, A and B). These data suggest that SPP protease family proteins maintain the high molecular weight complex structure in the DDM-solubilized condition.

To examine whether high molecular weight SPP complex is composed solely of SPP polypeptides or incorporates other components, we overexpressed 3FSPP (Fig. 2A) by the recombinant baculovirus/Sf9 cell system. 3FSPP polypeptide, which migrated at 50 kDa as a monomeric form, was detected as a heat-sensitive, SDS-resistant dimer on SDS-PAGE (19), was detected at 400 kDa on BN-PAGE (supplemental Fig. S1, A and B). These data suggest that SPP protease family proteins maintain the high molecular weight complex structure in the DDM-solubilized condition.

We further purified the DDM-solubilized Sf9 cell lysates by affinity column using anti-FLAG antibody (Fig. 2D). Silver staining and immunoblot analysis of each fraction revealed that the major polypeptides in eluate fraction migrated at 50 and 100 kDa, corresponding to the monomer and dimer of 3FSPP, respectively. Next we separated the purified 3FSPP by size-exclusion gel chromatography (SEC) (Figs. 2E and supplemental Fig. S2A). 3FSPP polypeptides were mainly detected at the fractions corresponding to ~250–450 kDa. Silver staining revealed that these fractions contained exclusively 3FSPP polypeptides. Moreover, all samples containing 3FSPP showed the SPP activity to cleave myc-Prl-PP-FLAG in vitro, suggesting that the high molecular weight complexes were composed only of proteolytically active 3FSPP (Fig. 2F and supplemental Fig. S2A). 3FSPP molecules were also subjected to BN-PAGE (Fig. 3B). DDM-solubilized 3FSPP was predominantly migrated as a 240-kDa high molecular mass complex similarly to endogenous SPP. In addition, 440- and 700-kDa complexes containing 3FSPP were detected. No additional major bands appeared between the 240-, 440-, and 700-kDa complexes, but three additional minor bands emerged below 240 kDa with an increased loading amount. Notably, these bands migrated as a monomer (60 kDa), dimer (120 kDa), and trimer (180 kDa) of 3FSPP, suggesting that the 240-kDa complex corresponds to a tetrameric complex of 3FSPP. Mutant

FIGURE 2. Biochemical characterization of recombinant 3FSPP expressed in Sf9 cells. A, schematic depiction of 3FSPP with predicted membrane topology. Catalytic aspartates are shown by stars. The black circles represent glycosylation. Ratios of each domain (i.e. luminal, transmembrane (TMD), and cytosolic domains), based on the composition of predicted amino acids, are indicated at the right. B, immunoblot analysis of lysates of Sf9 cells infected with recombinant baculovirus. Samples were loaded with (+) or without (−) pre-boiling (Boil). A heat-sensitive SDS-resistant dimer and monomer of 3FSPP are indicated by the black arrowheads and black arrows, respectively. The asterisks denote nonspecific bands. C, photoaffinity labeling experiment of infected Sf9 cell lysates with L-852,646, and L-852,505. Both probes successfully labeled 3FSPP, as indicated by the monomer (black arrows) as well as the dimer (black arrowheads), and the labeling was completely eliminated by the coincubation with the parent compound L-685,458 (comp.). D, affinity purification of 3FSPP from DDM-solubilized Sf9 cell lysates using an anti-FLAG antibody column. The arrows and arrowheads indicate the monomer and dimer of 3FSPP, respectively. E, immunoblot analysis of affinity-purified 3FSPP separated by SEC using anti-FLAG M2 antibody. The arrow and arrowhead indicate the monomer and the dimer, respectively. Fractions (Fr.) containing SEC markers are represented by the vertical white arrowheads below the upper panel. F, proteolytic activity of purified 3FSPP in vitro. SPP substrate (black arrowhead) was cleaved to generate endoproteolytic product (white arrowhead) by coincubation at 37 °C (Rxn (+)) with infected Sf9 cell lysates, eluate of anti-FLAG antibody column (B), and fraction 25 of SEC (C).
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FIGURE 3. Three-dimensional reconstruction of purified recombinant 3FSPP. A, silver staining of affinity-purified 3FSPP separated by SEC. The arrows and arrowheads indicate the monomer and the dimer, respectively. Fractions (Fr.) containing SEC markers are represented by the white arrowheads below the upper panel. B, BN-PAGE analysis of affinity-purified 3FSPP. DDM-solubilized 3FSPP proteins were mainly detected as tetramers (arrow) and dimeric and trimeric tetramers (black arrowheads). Note that an increase in loading amount resulted in the appearance of the monomer, dimer, and trimer of 3FSPP (white arrowheads). C, electron microscopic observation of negatively stained fraction 27 in panel A. Scale bar represents 400 Å. D, electron microscopic observation of a negatively stained affinity-purified 3FSPP fraction coincubated with colloidal gold-conjugated anti-FLAG antibody. The scale bar represents 400 Å. E, electron microscopic observation of negatively stained fraction 21 in panel A. The scale bar represents 400 Å. F, raw images of SPP with different Euler angles (row 1) are compared with the corresponding two-dimensional averages (row 2), the surface views of the three-dimensional reconstruction (row 3), and the reprojections of the three-dimensional reconstruction (row 4) along the corresponding Euler directions. They are consistent through the reconstruction. The scale bar represents 100 Å. G and H, surface and sections of reconstructed 3FSPP. Horizontal slices perpendicular to the four-fold symmetric axis show the bullet-shaped molecule. Representative raw images are presented in panel A, D, G, and H. Reprojections of the three-dimensional reconstruction (row 4), and with surface representations and reprojections (Fig. 3F, third and fourth rows). Reprojections from the final volume are consistent with raw images and class averages, reflecting successful reconstruction from the original particle images. A plot of the Euler angles of the 117 adopted class averages (supplemental Fig. S3A) showed that SPP is almost randomly oriented on the grid surface.

3FSPP carrying the protease-inactive D219A mutation (6) was also detected as a 240-kDa complex on BN-PAGE, suggesting that SPP forms the high molecular mass complex irrespective of the enzymatic activity (supplemental Fig. S2C). These data suggest that active SPP was purified as a tetramer in the DDM-solubilized condition. Moreover, appearance of monomer and trimer species on BN-PAGE indicates that the tetrameric assembly is not a dimeric form of the SDS-resistant dimer. 440- and 700-kDa complexes may represent oligomeric forms of the tetramer (i.e. octamer and dodecamer, respectively). The sizes of DDM-solubilized native SPP (monomer, 45 kDa; complex, 200 kDa), dSPP (monomer, 40 kDa; complex, 180 kDa), and tagged SPP/L2b (monomer, 90 kDa; complex, 400 kDa) are also consistent with tetramer formation, supporting the notion that the formation of a tetrameric complex is a common characteristic of the SPP family proteins.

Electron Microscopy and Three-dimensional Reconstruction of SPP—We then negatively stained the purified 3FSPP particles with uranyl acetate and viewed them by electron microscopy. In the fractions 26/27 (corresponding to 250 kDa from standard proteins) separated by SEC (Fig. 3, A and B), variously shaped particles of a uniform size were observed (Fig. 3C). These particles were labeled by colloidal gold-conjugated anti-FLAG antibody, indicating the 3FSPP tetramer (Fig. 3D). The dimeric form of the particles, each of which also has the same dimensions, was observed in the 450-kDa fractions 20/21 of SEC (corresponding to 450 kDa from standards) (Fig. 3E), supporting the idea that the 440-kDa complex in BN-PAGE corresponds to the dimeric form of the 3FSPP tetramer. Most particles were rhomboid- or square-shaped with round corners (Fig. 3C). The variation in shape was interpreted to reflect different orientations of the same molecule on the grid. The square-shaped particles seemed to imply top views of the tetrameric form; the rhombuses would be side views. For three-dimensional reconstruction of the SPP molecule, image analysis was performed using our SPINNS program and IMAGIC V (33–37). The final reconstruction included 4,232 particles, 90.2% of all the selected images. Representative raw images are presented (Fig. 3F, first row), with their corresponding class averages (Fig. 3F, second row) and with surface representations and reprojections (Fig. 3F, third and fourth rows). Reprojections from the final volume are consistent with raw images and class averages, reflecting successful reconstruction from the original particle images. A plot of the Euler angles of the 117 adopted class averages (supplemental Fig. S3A) showed that SPP is almost randomly oriented on the grid surface.
According to the Fourier shell correlation (FSC) function (41), 22 Å, if FSC > 0.5, was used as the resolution criterion (supplemental Fig. S3B).

For surface representation, the three-dimensional map was contoured at an isosurface containing a volume corresponding to 327 kDa: 182% of the tetrameric SPP mass (179 kDa) calculated from the amino acid composition. The additional volume seems attributable to glycosylation (4, 12, 22) and the attached lipids and detergents. The surface representation depicts a bullet-shaped molecule with a pointed bottom tip and a boat-tail top (Fig. 3G). Viewed from the top, SPP is a square with round corners: 100 × 85 Å. The height estimated from the side views is 130 Å. The structure has four low density interior regions connected to exterior openings, which are capped by a plug-like structure at the top (Fig. 3H and supplemental Fig. S3C). Moreover, a cleft-like concave domain for the tetramer formation of SPP, which is the counterpart domain of the C terminus, is responsible for tetramer formation. To confirm the topology of the 3FSPP particle, purified 3FSPP was cocentrated with anti-FLAG antibody and observed by electron microscopy. The particles were labeled with antibodies near the corners of the large domain (Fig. 3, D and I), suggesting that the boat-tail domain faces the luminal side and the tip locates at the cytosolic side. Taken together, single particle analysis revealed that the purified 3FSPP tetramer forms a bullet-shaped structure with a central chamber.

**N-terminal Region of SPP Is Responsible for Tetrameric Assembly**—It was reported that the recombinant C-terminal region of SPP containing five transmembrane domains with the catalytic aspartate was able to reconstitute the substrate binding as well as proteolytic activity in vitro and was present as a monomer (43). We thus hypothesized that the remaining N-terminal half of SPP, which is the counterpart domain of the C terminus, is responsible for tetramer formation. To examine this, we first purified the N-terminal region of 3FSPP (3FSPP/NT; truncated human SPP (1–191 amino acids) fused with 3×FLAG tag at its N terminus) overexpressed in Sf9 cells (Fig. 4A and supplemental Fig. S4). In addition to a monomer (30 kDa), an SDS-resistant homodimer of 3FSPP/NT was detected similarly to the SPP holoprotein (Fig. 4B). Recombinant 3FSPP/NT polypeptides were also detected as 130- and 260-kDa complexes on BN-PAGE, corresponding to the tetramer and the dimeric tetramer, respectively (Fig. 4C). Purified 3FSPP/NT was also observed as a half-size particle of the 3FSPP holoprotein in negatively stained images (Fig. 4D). These data strongly suggested that the N-terminal region is a scaffold domain for the tetramer formation of SPP.

To examine the physiological relevance of the tetramer formation of SPP within cells, we overexpressed dSPP/NT, which encodes the truncated dSPP (1–200 amino acids) equivalent to human 3FSPP/NT (Fig. 5, A and B, and supplemental Fig. S4). Consistent with the putative scaffolding function observed in 3FSPP, overexpressed dSPP/NT was co-immunoprecipitated with endogenous dSPP (Fig. 5C). Next we analyzed the possible function of dSPP/NT using a chemical biology approach. SPP utilizes two functional sites for the intramembrane cleavage, i.e. the initial substrate binding site and the catalytic site, which are directly targeted by the helical peptide- and the transition state analog-type inhibitors, respectively (8, 22, 31). A previous report indicated that both sites were present in the recombinant C-terminal fragment of dSPP (43). To test whether the dSPP/NT polypeptide harbors enzymatically functional sites, we performed a photoaffinity labeling experiment using the helical peptide- and the transition state analog-based photoprobes (pep.11-Bt and 31C-Bpa, respectively) (25, 44, 45). Consistent with the previous results, both wild-type dSPP and dSPP/ D228A were labeled by pep.11-Bt, whereas 31C-Bpa was bound only to wild-type dSPP, suggesting that the initial substrate binding site was formed irrespective of the endoproteolytic activity of dSPP (Fig. 5D). However, no labeling of dSPP/NT was observed by either of the photoprobes, indicating that dSPP/NT does not harbor a functional site for the enzymatic activity. We then examined the effect of dSPP/NT overexpression on the proteolytic activity in cells. dSPP activity was detected by the transcriptional activation of the ERSE-driven firefly luciferase (26), which was mediated by specific intramembrane cleavage of the recombinant SPP substrate (SPPsub) (22). We found that the coexpression of dSPP with ERSE reporter and SPPsub in S2 cells increased luciferase activity, which was decreased by the coexpression of an aspartate mutant dSPP, suggesting that the reporter assay is applicable to dSPP in S2 cells similarly to mammalian cells. Intriguingly, the overexpression of dSPP/NT inhibited
the proteolytic activity of endogenous dSPP in a similar manner to that observed by the expression of aspartate mutant dSPP, indicating that dSPP/NT functions as a dominant negative mutant (Fig. 5E). Moreover, the amount of the endogenous dSPP tetramer was reduced in cells expressing dSPP/NT (Fig. 5F). Considering that dSPP/NT lacked any mechanically functional sites for enzymatic activity, interaction of dSPP/NT with the endogenous dSPP disrupted the tetrameric assembly, thereby causing the dominant negative effect. Collectively, these data suggest that the N-terminal region of SPP is responsible for the tetrameric assembly, which might be the prerequisite for the intramembrane cleaving activity of SPP.

DISCUSSION

In this study, we analyzed the structure and biochemical character of SPP using in vitro as well as cell-based assays. We found that SPP forms a bullet-shaped tetramer with a large interior chamber. The tetrameric assembly was conserved among the SPP and SPP-like protease (SPPL) family proteins and was mediated by its N-terminal region. Overexpressed SPP/NT was incorporated into the SPP complex and inhibited

FIGURE 5. Effect of the overexpression of dSPP/NT on enzymatic activity. A, schematic depiction of dSPP/NT. B, immunoblot analysis of S2 cells expressing dSPP-V5 (white arrowheads) and dSPP/NT (black arrowheads). Endogenous dSPP is indicated by the arrow. C, immunoprecipitation of S2 cells expressing dSPP-V5 (white arrowheads) and dSPP/NT (black arrowheads). Endogenous dSPP and dSPP/NT are indicated by the arrow and black arrowheads, respectively. D, photoaffinity labeling experiments using S2 cell lysates expressing wild-type dSPP, dSPP/D228A, or dSPP/NT. Endogenous and exogenous dSPP and dSPP/NT were shown by the arrows, white arrowheads, and black arrowheads, respectively. Note that coincubation with the parent compound (comp.; pep.11 for pep.11-Bt, 31C for 31C-Bpa) completely eliminated the labeling. E, cell-based dSPP activity assay using S2 cells expressing dSPP mutants (n = 4, mean ± S.E., *, p < 0.05, as compared with mock). F, the amount of dSPP tetramer in DDM-solubilized S2 cells expressing dSPP-V5 (white arrowhead) and dSPP/NT (black arrowhead) was analyzed by BN-PAGE. Endogenous dSPP is indicated by an arrow (middle panel). The levels of endogenous Psn polypeptides (white arrow) are used as a loading control (lower panel). Antibodies used are indicated below the lanes.
the enzymatic activity in living cells, implying that the tetramer formation is the prerequisite for the proteolytic activity of SPP.

It was reported that human SPP proteins formed a SDS-resistant dimer (40). In contrast to mammalian SPP proteins, dSPP was solely detected as a monomer in SDS-PAGE (Fig. 1B) (43). However, DDM-solubilized human SPP, SPPL2b, and dSPP polypeptides were mainly detected as a tetramer complex on BN-PAGE. Thus, the tetramer formation is mediated by a common molecular mechanism, and the binding mode of the tetramer should be distinct from the formation of the SDS-resistant dimer observed in human SPP or SPPL2b. SDS-resistant dimer formation is not a critical mechanistic feature; rather, it appears to be an artificial phenomenon in human SPP caused by SDS. We also found that the N-terminal half is sufficient for the tetrameric assembly of SPP, although the precise mode of interaction still remains unknown. However, SPP/SPPL proteins were never cofractionated with other member proteins of the SPP family (19, 31), suggesting a specific mechanism of recognition and interaction behind homo-oligomerization. Very recently, 200-, 400-, and 600-kDa complexes containing SPP and its substrates were reported, in accordance with our results (46). EM showed a dimer form of the tetramer in SEC fractions 20 and 21, suggesting that the tetramer is an essential and minimal form of functional SPP. Further analyses will be needed to identify the critical domain(s) for the tetramerization.

A series of x-ray crystallographic studies revealed that the serine- and metalloprotease-types of I-CLiPs, e.g. rhomboid and S2P, respectively, harbor the active site residues within the hydrophilic cleft in the lipid bilayer (47). Moreover, we have biochemically shown that PS has the catalytic pore structure (48), suggesting that the hydrophilic interior structure of a catalytic site is a common feature essential to the intramembrane cleaving mechanism of I-CLiPs. Here, we analyzed the structure of SPP using single particle analysis and found that the SPP tetramer has a bullet-like structure with low density internal regions, to which the concaves near the boat-tail domain were connected. The boat-tail domain with its plug-like structure was predominantly labeled by an antibody targeting the N terminus, suggesting that SPP also has a hydrophilic chamber accessible from the luminal side (Fig. 6A). Intriguingly, the plug-like structure near the catalytic site was also predicted in the cytosolic side of PS (48). Considering the inverse topology of SPP as compared with PS (42), the four chambers within the SPP tetramer and the plug-like structures at the predicted luminal side represent the hydrophilic catalytic sites of SPP within the lipid bilayer, similarly to those of PS. The concaves connected to the chambers may represent the substrate entry sites. Furthermore, considering the previous study reporting that the C-terminal region of SPP restored proteolytic function in the monomeric state (43), we speculate that the N-terminal region functions as a scaffold for tetramer formation located at the center of the bullet-like structure. This may explain why a single anti-FLAG antibody bound to one tetramer despite high binding efficacy. Collectively, these data suggest that SPP forms a bullet-like tetramer with its N terminus at the center, whereas the enzymatically active C terminus is located as an outer ring (Fig. 6B). Nevertheless, further fine structural analyses, e.g. single particle reconstruction using cryo-EM or x-ray crystallography, will be needed to clarify the precise structure and function relationships of SPP.

Overexpression of dSPP/NT inhibited the activity of endogenous dSPP similarly to that of the catalytic site mutant. In accordance with previous studies of the C-terminal region of SPP (43), our chemical biology approach revealed that dSPP/NT contains neither the initial substrate binding site nor the catalytic site, suggesting that the inhibitory effect of dSPP/NT is independent of the proteolytic machinery (i.e. defects in catalytic function or capturing of substrates). In addition, the possibility of a nonspecific hydrophobic interaction of the substrate with dSPP/NT was excluded because nonspecific labeling of dSPP/NT with photoprobes, including a highly hydrophobic helical peptide, was not detected. Rather, we found that the amount of dSPP tetramer was reduced in cells expressing dSPP/NT, which is capable of binding to the holoprotein, suggesting that the interaction of dSPP/NT affected the tetramer formation of the endogenous dSPP (Fig. 6B). These results are totally different from those observed with PS, which is a homologous protease; exogenous N- or C-terminal fragments (NTF or CTF, respectively) of PS failed to be incorporated into a functional complex, nor did it affect the levels or activity of endogenous γ-secretase (49, 50). Moreover, we failed to overexpress the C-terminal region of dSPP with or without dSPP/NT (data not shown), whereas the coexpression of PS NTF and CTF reconstructed the functional γ-secretase complex (51). Thus, the tetrameric assembly of SPP with its N-ter-
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terminal region is a unique feature of the SPP/SPPL family I-CLiPs. Oligomer formation is the prerequisite for the function of several channels, receptors, and transporters. In particular, the tetramer formation of aquaporin is critical to its water permeability and ion selectivity (52–54), whereas each aquaporin subunit has a water channel in the center. We speculate that the interaction of dSPP/NT resulted in a conformational change of each subunit that affects the formation and/or the stability of the DSP tetramer, thereby causing the dominant negative effect (Fig. 6B). These results also imply that the N-terminal region is the scaffold domain for the formation of the enzymatically active DSP tetramer.

In sum, we have revealed that SPP forms a bullet-shaped tetramer with its N-terminal region in the proteolytically active state. However, we were not able to fully characterize the structure of SPP in the membrane-embedded state. Further fine structural analyses, e.g. reconstruction of SPP in the presence of lipids and x-ray analysis of its Type I three-dimensional crystals composed of two-dimensional crystal layers in lipid, should eventually identify the mechanism by which the SPP complex recognizes the substrates, which in turn leads to the entrance of the substrates into the active site.

Acknowledgments—We are grateful to Drs. T. Fukuyama and S. Yokoshima (The University of Tokyo), N. Umezawa, T. Higuchi (Nagoya City University), R. Flucher, and C. Haass (Ludwig-Maximilians-University Munich), E. I. Rogaev (University of Massachusetts Memorial University Munich), E. I. Rogaev (University of Massachusetts Amherst), T. Fukuyama and S. Hashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) *Nat. Cell Biol.* 422, 1671–1679.

Sato, T., Ananda, K., Cheng, C. I., Suh, E. J., Narayanan, S., and Wolfe, M. S. (2007) *J. Biol. Chem.* 282, 38040–38046.

Ogura, T., Mio, K., Hayashi, I., Miyashita, H., Fukuda, R., Kornilova, A., Hyman, B. T., Perrimon, N., and Wolfe, M. S. (2003) *J. Biol. Chem.* 279, 20172–20179.

Haraus, G., and van Heel, M. (1986) *Optik* 73, 146–156.

Friedmann, E., Lemberg, M. K., Weihofen, A., Dev, K. K., Dengler, U., Kopan, R., Kornilova, A., Hyman, B. T., Perrimon, N., and Wolfe, M. S. (2003) *FASEB J.* 17, 79–81.

Schul, B., Kapp, K., Sinning, I., and Dobberstein, B. (2010) *Biochem. J.* 427, 523–534.

Urban, S., and Shi, Y. (2008) *Curr. Opin. Struct. Biol.* 18, 432–441.

REFERENCES

1. Wolfe, M. S. (2009) *J. Biol. Chem.* 284, 13969–13973.
2. Flucher, R., Steiner, H., and Haass, C. (2009) *J. Biol. Chem.* 284, 13975–13979.
3. Golde, T. E., Wolfe, M. S., and Greenbaum, D. C. (2009) *Semin. Cell Dev. Biol.* 20, 225–230.
4. Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K., and Martoglio, B. (2002) *Science* 296, 2215–2218.
5. Kornilova, A. Y., Das, C., and Wolfe, M. S. (2003) *J. Biol. Chem.* 278, 16470–16473.
6. Weihofen, A., Lemberg, M. K., Friedmann, E., Rueeger, H., Schmitz, A., Paganetti, P., Rovelli, G., and Martoglio, B. (2003) *J. Biol. Chem.* 278, 16528–16533.
7. Nyborg, A. C., Ladd, T. B., Jansen, K., Kadd, T. B., Fauq, A., and Golde, T. E. (2004) *J. Biol. Chem.* 279, 43148–43156.
8. Ogura, T., Mio, K., Hayashi, I., Miyashita, H., Fukuda, R., Kopan, R., Kornilova, A., Hyman, B. T., Perrimon, N., and Wolfe, M. S. (2003) *J. Biol. Chem.* 279, 41670–41676.
9. Tomita, T., Maruyama, K., Saito, T. C., Kume, H., Shinozaki, K., Tokuihiro, S., Capelli, A., Walter, J., Grünberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 2025–2030.
10. Sato, T., Nyborg, A. C., Iwata, N., Diehl, T. S., Saito, T. C., Golde, T. E., and Wolfe, M. S. (2006) *Biochemistry* 45, 8649–8656.
11. Frank, J. (2006) *Three-Dimensional Electron Microscopy of Macromolecular Assemblies: Visualization of Biological Molecules in Their Native State*, Oxford University Press, New York.
12. van Heel, M., Haraus, G., Orlova, E. V., Schmidt, R., and Schatz, M. (1996) *J. Struct. Biol.* 116, 17–24.
13. Ogura, T., and Sato, C. (2004) *J. Struct. Biol.* 145, 354–358.
14. Ogura, T., and Sato, C. (2004) *J. Struct. Biol.* 145, 63–75.
15. Ogura, T., and Sato, C. (2006) *J. Struct. Biol.* 151, 371–386.
16. Ogura, T., Iwasaki, K., and Sato, C. (2003) *J. Struct. Biol.* 143, 185–200.
17. Penczek, P., Nitschke, N., and Frank, J. (1992) *Ultramicroscopy* 40, 33–53.
18. Penczek, P. A., Grassucci, R. A., and Frank, J. (1999) *Ultramicroscopy* 53, 251–270.
19. 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.
20. Haraus, G., and van Heel, M. (1986) *Optik* 73, 146–156.
21. Friedmann, E., Lemberg, M. K., Weihofen, A., Dev, K. K., Dengler, U., Rovelli, G., and Martoglio, B. (2004) *J. Biol. Chem.* 279, 50790–50798.
22. Narayanan, S., Sato, T., and Wolfe, M. S. (2007) *J. Biol. Chem.* 282, 20172–20179.
23. Das, C., Berezovska, O., Diehl, T. S., Genet, C., Buldyrev, I., Tsai, J. Y., Hyman, B. T., and Wolfe, M. S. (2003) *J. Am. Chem. Soc.* 125, 11794–11795.
24. Michell, C. A., Esler, W. P., Kimberly, W. T., Jack, C., Berezovska, O., Kornilova, A., Hymann, B. T., Perrimon, N., and Wolfe, M. S. (2003) *FASEB J.* 17, 79–81.
25. Sato, T., Nyborg, A. C., Iwata, N., Diehl, T. S., Saito, T. C., Golde, T. E., and Wolfe, M. S. (2006) *J. Biol. Chem.* 281, 39515–39523.
26. McLauchlan, J., Lemberg, M. K., Hope, G., and Martoglio, B. (2002) *EMBO J.* 21, 3980–3988.
27. Takasugi, N., Takahashi, Y., Morohoshi, Y., Tomita, T., and Iwatsubo, T. (2002) *J. Biol. Chem.* 277, 50198–50205.
28. Nyborg, A. C., Jansen, K., Ladd, T. B., Fauq, A., and Golde, T. E. (2004) *J. Biol. Chem.* 279, 30951–30956.
29. Takashashi, Y., Morohoshi, Y., Tomita, T., and Iwatsubo, T. (2002) *J. Biol. Chem.* 277, 30951–30956.
30. Tomita, T., Maruyama, K., Saito, T. C., Kume, H., Shinozaki, K., Tokuihiro, S., Capelli, A., Walter, J., Grünberg, J., Haass, C., Iwatsubo, T., and Obata, K. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 2025–2030.
31. Sato, T., Nyborg, A. C., Iwata, N., Diehl, T. S., Saito, T. C., Golde, T. E., and Wolfe, M. S. (2006) *Biochemistry* 45, 8649–8656.
32. Frank, J. (2006) *Three-Dimensional Electron Microscopy of Macromolecular Assemblies: Visualization of Biological Molecules in Their Native State*, Oxford University Press, New York.
48. Sato, C., Morohashi, Y., Tomita, T., and Iwatsubo, T. (2006) *J. Neurosci.* 26, 12081–12088
49. Tomita, T., Tokuhiro, S., Hashimoto, T., Aiba, K., Saito, T. C., Maruyama, K., and Iwatsubo, T. (1998) *J. Biol. Chem.* 273, 21153–21160
50. Shirotani, K., Takahashi, K., and Tabira, T. (1999) *Neurosci. Lett.* 262, 37–40
51. Laudon, H., Mathews, P. M., Karlström, H., Bergman, A., Farmery, M. R., Nixon, R. A., Winblad, B., Gandy, S. E., Lendahl, U., Lundkvist, J., and Näslund, J. (2004) *J. Neurochem.* 89, 44–53
52. Mathai, J. C., and Agre, P. (1999) *Biochemistry* 38, 923–928
53. King, L. S., Kozono, D., and Agre, P. (2004) *Nat. Rev. Mol. Cell Biol.* 5, 687–698
54. Buck, T. M., Wagner, J., Grund, S., and Skach, W. R. (2007) *Nat. Struct. Mol. Biol.* 14, 762–769