The γ-secretase membrane protein complex is responsible for proteolytic maturation of signaling precursors and catalyzes the final step in the production of the amyloid β-peptides implicated in the pathogenesis of Alzheimer disease. The incorporation of PEN-2 (presenilin enhancer 2) into a pre-activation intermediate, composed of the catalytic subunit presenilin and the accessory proteins APH-1 (anterior pharynx-defective 1) and nicastrin, triggers the endoproteolysis of presenilin and results in an active tetrameric γ-secretase. We have determined the three-dimensional reconstruction of a mature and catalytically active γ-secretase using single-particle cryo-electron microscopy. γ-Secretase has a cup-like shape with a lateral belt of ~40–50 Å in height that encloses a water-accessible internal chamber. Active site labeling with a gold-coupled transition state analog inhibitor suggested that the γ-secretase active site faces this chamber. Comparison with the structure of a trimeric pre-activation intermediate suggested that the incorporation of PEN-2 might contribute to the maturation of the active site architecture.

Alzheimer disease is a prevalent cause of dementia in adults (1). The neuropathological hallmarks of Alzheimer disease include the presence of senile plaques and neurofibrillary tangles throughout the cerebral cortex and hippocampus and the loss of neurons in these specific brain areas (2). Senile plaques are composed of dystrophic neurites surrounding extracellular aggregates of amyloid β-peptides (Aβ) that are derived from the sequential proteolytic degradation of amyloid precursor proteins (APPs) to produce Aβ peptides that range from 34 to 42 amino acids in length (3, 4). APP is first cleaved in its ectodomain segment by BACE1, and the resulting C-terminal membrane-associated derivatives are subsequently hydrolyzed within their transmembrane domain (TMD) by γ-secretase. This intramembrane proteolytic activity of γ-secretase is critical in other cellular processes, such as the activation of Notch and ErbB4-dependent signaling (4, 5), and in the processing of a growing list of additional type I integral membrane proteins, including APP-like proteins, E-cadherin, CD44, and lipoprotein receptor-related protein (reviewed in Ref. 6).

Reconstitution experiments in Saccharomyces cerevisiae have demonstrated that the integral membrane proteins presenilin (PS; 52.7 kDa), nicastrin (NCT; 78.4 kDa if not glycosylated), APH-1 (anterior pharynx-defective 1; 29 kDa), and PEN-2 (presenilin enhancer 2; 12 kDa) are essential and sufficient for γ-secretase activity (7). It is now widely accepted that PS, a polypeptide with nine predicted TMDs (8) and two absolutely conserved catalytic aspartates in the hydrophobic region of adjacent TMD6 and TMD7 (9), is the catalytic subunit of γ-secretase. In fact, PS is now considered to be the founding member of diasparyl intramembrane proteases, which are also found in other eukaryotes (10, 11) and in archaea (12). In vivo, PS undergoes endoproteolysis in the intracellular loop between TMD6 and TMD7, resulting in N- and C-terminal fragments that remain stably associated (13, 14). The endoproteolysis of PS is a prerequisite for γ-secretase activity (13). The functional roles of the remaining components of γ-secretase are not fully established, but important information has emerged. The ectodomain of the type I membrane protein NCT plays a critical role as a substrate receptor (15) and serves to recognize the N terminus of the membrane-tethered stubs generated by ectodomain shedding of the various substrates described above. PEN-2, the smallest of the γ-secretase subunits, is obligatory for mediating PS endoproteolysis (16). Structural information on...
γ-secretase is scanty. Recent three-dimensional reconstructions of γ-secretase using single-particle electron microscopy have provided some insights (17, 18).

The in vivo assembly and maturation of γ-secretase are coordinately regulated (19) and have been shown to occur in a stepwise fashion (20). First, APH-1 and NCT appear to serve as a “scaffold” onto which PS binds to from stable but catalytically inactive complexes in the endoplasmic reticulum (16, 21). Next, the association of PEN-2 with this trimeric pre-activation intermediate is essential for the endoproteolytic cleavage of PS and for conferring γ-secretase activity to the complex (16, 22–25). In addition, the incorporation of PEN-2 appears to correlate with the acquisition of complex N-linked oligosaccharide modifications within the NCT ectodomain (26). Following the assembly of the complex and the endoproteolysis of PS, the γ-secretase complex is exported from the endoplasmic reticulum to Golgi and post-Golgi membrane compartments (reviewed in Ref. 27). At a molecular level, TMD4 of APH-1 (28, 29), the C terminus of PEN-2 (30, 31), TMD of NCT (32–34), and the C terminus of PS appear to be necessary for γ-secretase assembly, whereas the binding of PEN-2 to TMD4 of PS appears to be essential for PS endoproteolysis (35, 36). The structural changes associated with the incorporation of PEN-2 into the trimeric pre-activation intermediate have not been visualized.

Here, we used cryo-EM and image processing to describe the architecture of γ-secretase under conditions in which the enzyme displays physiological-like activity. A gold-labeled transition state analog was used to determine the approximate location of the γ-secretase active site. Comparison with a three-dimensional reconstruction of a trimeric pre-activation intermediate that lacks PEN-2 provides structural insight into the activation of γ-secretase.

**EXPERIMENTAL PROCEDURES**

*Constructs and Cell Lines*—Human HEK293S cells stably expressing TAP-PS1, NCT-CT11, APH-1, and PEN-2-CT11 have been described previously (37). PEN-2 knockdown 293S cells were generated by a stable transfection of TAP-PS1, NCT, and APH-1. Transfected cell lines were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. For suspension culture, HEK293S cells were grown in IS GRO™ medium (Irvine Scientific) supplemented with 10% FBS, 10 mM l-glutamine, 1% penicillin/streptomycin, and 200 µg/ml hygromycin at 37 °C.

**γ-Secretase Purification**—The purification of γ-secretase from HEK293S cells has been described previously (37). Briefly, HEK293S cells overexpressing the γ-secretase components were harvested and homogenized, and the post-nuclear supernatant fraction was collected by centrifugation. The membrane pellet was solubilized in 50 mM sodium citrate (pH 6.4), 1% CHAPSO, 10% glycerol, 1 mM EDTA, and 1 X protease inhibitor on ice for 2 h. The solubilized membrane protein preparation was then incubated with IgG-Sepharose beads (GE Healthcare) that had been pre-equilibrated with 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.5% CHAPSO. Tobacco etch virus protease digestion was performed at 16 °C to elute γ-secretase from the beads, and this eluate was incubated with wheat germ agglutinin beads to capture the mature γ-secretase complex. The complex was then eluted with 500 mM glucosamine and incubated with calmodulin beads (Stratagene) that had been pre-equilibrated with 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM magnesium acetate, 2 mM CaCl2, 0.5% CHAPSO, and 10 mM β-mercaptoethanol. The complex was eluted with buffer A (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM magnesium acetate, 0.5% CHAPSO, and 5 mM EGTA) and concentrated.

**γ-Secretase Activity Assays**—An aliquot of 0.5–4 µl of purified γ-secretase was used to assay activity by incubation with recombinant C100-FLAG as a substrate (39). The reactions were terminated by adding radioimmune precipitation assay buffer and boiling for 5 min. The samples were centrifuged, and the AB peptides in the supernatant were quantified using an electrochemiluminescence (ECL)-based assay.

**Electron Microscopy and γ-Secretase Labeling**—For cryo-specimen preparation, a 2–4 µl aliquot of purified γ-secretase (20 nM) in buffer A was adsorbed for 30 s onto non-glue-discharged holey grids (QUANTIFOIL R 2/4 on copper/rhodium, 300 mesh) coated with thin carbon, followed by blotting and plunging into liquid ethane. The grids were transferred to a liquid nitrogen-cooled Gatan 626 cold stage, and low dose electron micrographs of specimens were imaged on a JEOL 2100F field emission gun transmission electron microscope at 200 kV. The images were recorded on a 2000 × 2000-pixel CCD camera at the equivalent calibrated magnification of ×78,473. Negatively stained specimens were prepared using 2% uranyl acetate.

For antibody labeling of γ-secretase, an aliquot of the enzyme (20 nM) was incubated with rabbit anti-NCT54 polyclonal antibody (an in-house antibody generated against amino acids 242–546 of the extracellular domain of NCT) (22), mouse anti-PS1 monoclonal antibody (Sigma), rabbit anti-PEN-2 polyclonal antibody (Sigma), or anti-APH-1 antibody (Sigma) on ice for 8 h. To these incubations was added a 40 µM final concentration of 5-nm gold-conjugated IgG secondary antibody (Sigma). For the active site labeling of γ-secretase, an aliquot of the enzyme (20 nM) was incubated for 1 h at 37 °C with a 2 µM final concentration of the active site-directed inhibitor B458-C (40). This compound consists of the γ-secretase inhibitor L-685,458 (41) coupled to a biotin moiety via a 39.4-Å linker. Subsequently, the inhibited sample was labeled with a 0.1 mM final concentration of 1.4-nm Nanogold®-streptavidin (Nanoprobes, Yaphank, NY) for 1 h at room temperature. Two microliters of this reaction mixture were applied to carbon-coated copper/rhodium grids (400 mesh) for 1 min. The specimen was partially blotted, and the grid was applied to the surface of a 40 µl droplet of gold developer reagent (Nanoprobes) for 20 s. Gold enhancing is necessary to increase the diameter of the gold particles for optimal visualization under negative stain (42). Under this technique, gold is deposited in a controlled and specific fashion around the initial gold particle, and the resulting 5–20 nm gold particles can be easily visualized under negative stain. The grid
was then rinsed with deionized water, stained with 6% ammonium molybdate and 2% trehalose at pH 8.3, and blotted.

**Data Processing and Volume Reconstruction**—To avoid biases generated during manual particle selection, we applied a strategy that involves automated particle selection, followed by statistical analysis, alignment, and classification using maximum likelihood approaches (43, 44). We have recently implemented this strategy to determine the three-dimensional reconstruction of a detergent-solubilized ~160-kDa membrane protein (45). An initial data set of 150,000 particles was automatically selected from 550 CCD images using EMAN (46). The software Xmipp (43, 44) was used to extract particles in 86 × 86 images, to normalize them, and to perform statistical analysis. Following normalization, ~10% of the initial images were discarded using purely statistical criteria. The contrast transfer function of the images was estimated using CTFFIND3 (47) and corrected using Bsoft (48). Subsequently, the particles were grouped into six different defocus groups to perform two-dimensional maximum likelihood classification in Fourier space using the Xmipp package. During this process, particle images with an irregular background, close neighbors, overlapping particles, and aggregates were discarded to yield a working data set of 40,000 particles.

To generate an initial random conical tilt template, 1720 pairs of negatively stained particles were selected from 0° and 50° tilted images collected with SerialEM (49). We carried out principal component analysis, hierarchical clustering, and multireference alignment classification with SPIDER. The Euler angles for 24 classes were evaluated and used to carry out a three-dimensional reconstruction and refinement for each class. Three volumes were then selected and merged. The random conical tilt volume was low pass-filtered at 30 Å and used as a template in reference-based alignment against the data set of 40,000 cryo-particles of γ-secretase. These initial alignment parameters were used in a second refinement procedure using FREALIGN (50). We performed 10 iterations of local refinement and three-dimensional reconstruction until convergence. The resolution of the final three-dimensional structure was calculated at 18 Å using the Fourier shell correlation (FSC) = 0.5 criterion of the FSC technique (51). A comparable approach was employed for the reconstruction of the trimeric pre-activation intermediate. In this case, the resolution of the final three-dimensional structure was calculated to be 22 Å.

**Single-tilt Axis Tomography**—Cryo-grids were prepared using γ-secretase that had been gold-labeled with the biotinylated inhibitor and gold-conjugated streptavidin (as described under “Electron Microscopy and γ-Secretase Labeling”). Single-tilt axis cryotomography data were collected at ~5-μm defocus. Images were collected using SerialEM from −60° to +60° at 3° intervals for a total of 40 images per tomogram. Gold was used as a fiducial marker to align the images. The images were processed with the software ETOMO following the flow diagram of the program (52).

**RESULTS**

**Expression and Purification of Enzymatically Active γ-Secretase Complexes**—TAP methods (53) have been successfully applied for the purification of protein complexes from eukaryotic cells. We chose to express human PS1 with a TAP tag appended to the N terminus of PS1 (TAP-PS1), as this strategy has given us excellent results recently (37). The TAP tag consisted of a duplicate of the IgG-binding site of protein A from *Staphylococcus aureus* (54) and a segment of calmodulin-binding protein separated by a specific tobacco etch virus protease cleavage site. Transient expression of TAP-PS1, NCT, APH-1, and PEN-2 in HEK293 cells led to TAP-PS1 endoproteolysis, a surrogate marker for γ-secretase activity (22), and the accumulation of high levels of ~55-kDa TAP-PS1 N-terminal fragment and ~20-kDa PS1 C-terminal fragment (supplemental Fig. S1A). The activity of transiently overexpressed γ-secretase complexes containing TAP-PS1 was examined (supplemental Fig. S1B, C and D) in immortalized fibroblasts lacking endogenous *PSEN1* and *PSEN2* (PS1/−/−/PS2/−/− cells) against substrates derived from APP and Notch and was found to be comparable with the activity exhibited by wild-type γ-secretase.

For γ-secretase purification, we stably coexpressed TAP-PS1, NCT, APH-1, and PEN-2 (supplemental Fig. S1A) in HEK293S cells. The level of expression of the exogenous γ-secretase components was 10-fold higher than that observed for endogenous γ-secretase components, in agreement with our previous study (22). The isolated membrane fractions were solubilized in 1% CHAPSO, and all subsequent purification steps and the EM analyses were carried out in 0.5% CHAPSO under conditions that preserve γ-secretase activity (39). The detergent-solubilized membrane fraction was first applied to IgG-Sepharose beads, and the bound γ-secretase was eluted by tobacco etch virus digestion. The eluate was then loaded onto wheat germ agglutinin-Sepharose beads to enrich for γ-secretase containing fully glycosylated NCT. In the last step of the purification, γ-secretase was bound to calmodulin-agarose beads in the presence of Ca2+ and eluted with EGTA. Western blot analysis (Fig. 1A) using antibodies specific for the γ-secretase subunits confirmed the presence of the PS1 N- and C-terminal fragments, PEN-2, and NCT in the purified complex. (The presence of APH-1 was not probed.) Silver-stained SDS-PAGE analysis of the concentrated final eluate suggested the γ-secretase preparation to be highly pure, with few detectable contaminants (Fig. 1B), indicating a considerable improvement over previous purification efforts (55, 56). The enzymatic activity of the purified complex was determined by an established *in vitro* assay (39). This assay employs a chimeric substrate, C100-FLAG, which encodes amino acids 596–695 of the 695-amino acid-long isoform of APP followed by a FLAG sequence, and ECL to quantify production of Aβ40 and Aβ42. The production of Aβ was fully inhibited by L-685,458 (Fig. 1C), a potent and selective transition state isosteric inhibitor (41) of γ-secretase activity, demonstrating that the observed activity was γ-secretase-specific. The Aβ42/Aβ40 ratio yielded by our detergent-solubilized γ-secretase (Fig. 1D) was similar to the ratio observed *in vitro* and in the conditioned medium of mammalian cells (57). The calculated γ-secretase-specific activity for production of Aβ40 and Aβ42 was 22.5 pM min⁻¹ and 4.9 pM min⁻¹, respectively.

**Cryo-EM and Three-dimensional Structure Calculation**—Fig. 2A displays a representative field view showing vitrified,
mostly globular particles of ~100 Å in diameter, although larger particles can also be observed. To be able to distinguish fully assembled γ-secretase particles from aggregates or artifac-tual oligomers, we used gold markers in combination with sub-unit-specific antibodies and an active site affinity probe (Fig. 2, B–F). The probe consisted of an established transition state analog γ-secretase inhibitor (L-685,458) (41) modified with a newly designed spacer arm that is conjugated to biotin. We have previously used this active site probe to successfully capture active γ-secretase (40). Cryo-images of γ-secretase that was incubated with the biotinylated inhibitor and with gold-conjugated streptavidin show particles marked with a single gold label (Fig. 2B). The labeled particles were comparable in shape and size with those shown in Fig. 2A. Further labeling studies using subunit-specific primary antibodies followed by the addition of a gold-conjugated secondary antibody confirmed the presence of each γ-secretase subunit in the particles (Fig. 2, C–F). The fact that we observed a single gold particle per complex suggests a 1:1:1:1 stoichiometry of the four individual components in active γ-secretase, in line with a previous report (58).

To calculate three-dimensional maps, we first generated an initial three-dimensional model by the random conical tilt method (59). We recorded a total of 1720 image pairs (at 0° and 50° tilt) at the same location of specimens stained with 2% ura-nyl acetate (supplemental Fig. S2A), and the image analysis and three-dimensional reconstruction were carried out with SPI- DER (60). This procedure yielded a starting volume that was low pass-filtered to 30 Å and employed as a template in refer-ence-based alignments by projection matching against a subset of 40,000 vitrified γ-secretase complexes. This subset of cryo-particles had been selected to display a high correlation in terms of size and background using maximum likelihood approaches (43, 44). Particles views were well distributed over all Euler angles. These initial alignment parameters were used in a sec-ond iterative refinement using FREALIGN. The calculated structure converged at a resolution of ~18 Å, as evaluated by an FSC curve at the 0.5 value (Fig. 2G). Back-projections of the final three-dimensional reconstruction correspond closely with experimental class averages, indicating consistency between the reconstructed structure and the particle data set (Fig. 2H).

As an independent quality control experiment, we performed a three-dimensional reconstruction using cryo-electron tomography (supplemental Fig. S3). Although the resolution that can be obtained by cryo-electron tomography for a particle such as γ-secretase is very limited, this technique does not suffer from biases in image classification and three-dimensional recon-struction. This is due to the fact that images of the same object

FIGURE 1. Purification of mature and enzymatically active γ-secretase. A, TAP of γ-secretase from HEK293S cells that stably overexpress TAP-PS1, NCT, APH-1, and PEN-2. The starting membrane solubilisate (Input), flow-through (FT), wash, and eluate from each step were probed by Western blotting against full-length TAP-PS1 and its endoproteolytic derivatives, NCT and PEN-2. Each fraction is indicated at the top. WGA, wheat germ agglutinin; NTF, N-terminal fragment; CTF, C-terminal fragment; CBP, calmodulin-binding protein. B, purified γ-secretase was analyzed by SDS-PAGE and visualized by silver staining. The bands corresponding to the γ-secretase components are indicated. C, the transition state analog inhibitor L-685,458 inhibited production of Aβ40 by purified γ-secretase. DMSO, dimethyl sulfoxide. D, purified γ-secretase catalyzed the in vitro conversion of the substrate C100-FLAG to both Aβ40 and Aβ42 products in a ratio analogous to that found in vivo.
are collected at small angular increments and used directly for three-dimensional reconstruction of this object (61). We prepared cryo-grids of \( /H9253\)-secretase labeled with the gold-labeled biotinylated inhibitor as a fiducial marker to align the tomogram sections. Because this transition state analog inhibitor reacts only with catalytically competent \( /H9253\)-secretase (40), this strategy ensured that our tomographic reconstruction was carried out only with active complexes. We collected single-tilt axis images from \( /H11002\) to \( /H11001\) taking an image every 3° under low dose conditions. Notwithstanding the characteristic missing wedge-dependent distortion and the expected lack of detail due to low signal to noise and possible radiation damage, the cryotomographic reconstruction at 35-Å resolution has comparable dimensions and shape relative to the single-particle electron microscopy map of \( /H9253\)-secretase. As an additional control, we carried out an initial three-dimensional reconstruction under negative stain of \( /H9253\)-secretase produced in the yeast \( \text{Pichia pastoris}\) (supplemental Fig. S4). This map also compares well with the cryo-map obtained for \( /H9253\)-secretase produced in \( \text{HEK293S}\) cells.

Description of the \( /H9253\)-Secretase Structure and Location of Its Active Site—Fig. 3 shows a set of views of the three-dimensional reconstruction of \( /H9253\)-secretase rendered at a threshold that includes a volume corresponding to a molecular mass (determined using an average protein density of 0.84 Da/Å\(^3\)) (62) of \( \sim 200\) kDa, corresponding to the mass of a \( /H9253\)-secretase complex with a 1:1:1:1 stoichiometry (as determined from the labeling experiments in Fig. 2). The map is 100 Å wide (\( x\) axis), 70 Å deep (\( y\) axis), and 105 Å tall (\( z\) axis). Several features of the map give us clues as to the most likely topology of the complex and the location of the NCT ectodomain. In a plane perpendicular to the \( z\) axis, a strong EM density surrounds the entire map as a continuous belt of \( \sim 40\)–\(50\) Å in height that encloses a chamber. The belt corresponds well with the average thickness of eukaryotic cellular membranes (63). Thus, it likely corresponds to the transmembrane region of the complex, in agreement with a similar density feature observed by Lazarov et al. (17). In addition, the distribution of mass outside of the plane of the membrane is very asymmetric, with most of the mass located on one side. Remarkably, a large domain (Fig. 3, marked with asterisks) covers most of the membrane surface and has a maximum height of \( \sim 50\) Å. This domain corresponds to a mass of \( \sim 100\) kDa, which is consistent with the mass of a glycosylated ectodomain of NCT. On the ectodomain region of NCT, a concave surface (marked with white arrows) that leads to a cavity within the proposed transmembrane region of the complex is clearly apparent. The cavity extends from the extracellular surface of the membrane, where it has maximum dimensions, past the membrane center, where it appears to lead to a small pore (marked with black arrows). A tantalizing possibility is that the active site of \( /H9253\)-secretase lies within this presumably water-accessible cavity.

To address this possibility, we undertook a strategy analogous to the one used above (Fig. 2B). In previous experiments,
we employed several biotinylated inhibitors to capture detergent-solubilized γ-secretase (40). These biotinylated inhibitors contained spacers of different lengths that were immobilized to streptavidin beads. Only when the length of the spacer arm connecting the inhibitor to the biotin moiety was >30.5 Å could the complex be labeled (40). We interpreted this observation as an indication that the active site of γ-secretase might be distant from the outside surface of the complex. Therefore, γ-secretase was incubated with a compound with a 39.4-Å spacer arm between the inhibitor and the biotin moiety (Fig. 4A). Nanogold-streptavidin, consisting of a 1.4-nm gold particle conjugated to streptavidin in a 1:1 stoichiometry, was then added to the mixture. Gold enhancing, commonly used to better visualize small Nanogold particles (42), was employed to image the particles under negative stain. Fig. 4B shows a gallery of projection images of γ-secretase marked with a gold-labeled transition state analog inhibitor. The gold particles can be clearly identified by their strong positive contrast. Only one gold label per complex could be observed, suggesting a 1:1 stoichiometry between the inhibitor and γ-secretase. Single-particle averaging of labeled complexes (100 particles) indicated that the gold label was located in the periphery of the complex, presumably because of the flexibility conferred by the long (39.4 Å) spacer arm between the inhibitor and the label. We note that the length of the linker precludes a direct identification of the location of the active site; however, the most likely position for the active site is at least 39.4 Å away from the surface of the complex, in agreement with our substrate capture experiments. In our map, this position corresponds to the region where the internal cavity lies.

Comparison of γ-Secretase with Its Trimeric Pre-activation Intermediate—PS, APH-1, and NCT form stable but catalytically inactive complexes in the endoplasmic reticulum (16). The incorporation of PEN-2 into this trimeric pre-activation intermediate is essential for endoproteolytic cleavage of PS and for conferring γ-secretase activity to the complex (16, 22, 23). We performed a three-dimensional reconstruction of a trimeric pre-activation intermediate of γ-secretase (composed of PS, APH-1, and NCT) that was imaged under negative stain. To purify this intermediate, we generated PEN-2 knockdown cells harboring human TAP-PS1, NCT, and APH-1 (supplemental Fig. S5A). In the absence of PEN-2, the C-terminal fragments of APP accumulated as a result of the lack of γ-secretase activity. As expected, PS1 remained in its full-length form (supplemental Fig. S5B). The purified trimeric complex was analyzed by Western blotting to confirm the presence of the three components, and the purity of the purified complex was assessed by silver staining (supplemental Fig. S5C). Fig. 5A displays a representative field view showing abundant globular particles with a diameter of ~100 Å. For three-dimensional reconstruction, we employed a procedure comparable to the one described for mature γ-secretase. After statistical analysis and two cycles of two-dimensional classification by maximum-likelihood in Fourier space (MLF2D), we isolated 10,000 particle images that displayed a very high correlation in terms of size and background. This subset of particle images was employed for three-dimensional reconstruction and refinement. The final structure was determined to a resolution of 22 Å based on the 0.5 criterion of the FSC curve (supplemental Fig. S2C). Fig. 5B shows a comparison of the trimeric pre-activation intermediate...
with γ-secretase. The trimeric pre-activation intermediate has overall dimensions similar to those of γ-secretase, consistent with the fact that both complexes have a mass difference of just 12 kDa (corresponding to PEN-2; ~7% of the total mass). Because of this small difference in size, we could not locate the position of PEN-2 in the complex. Although both structures are comparable, indicating the absence of large conformational changes upon the incorporation of the activating subunit PEN-2 into the pre-activation intermediate, there are interesting differences in relevant regions. There appears to be a rearrangement of the region we have attributed to NCT (Fig. 5C, marked with black arrows), which is most evident when we cut through the structure. Another significant difference is the observed widening (marked with a black arrow) of the internal chamber γ-secretase, relative to the trimeric intermediate (Fig. 5C), and the opening of a low density region that faces the outside of the complex.

**DISCUSSION**

γ-Secretase is a prominent member of an emerging family of membrane enzymes that have in common the ability to hydrolyze peptide bonds within transmembrane helices (64). Structural insight on γ-secretase is pivotal in unraveling the functional details of intramembrane proteolysis in biology and disease. Our cryo-EM γ-secretase structure has important similarities and significant differences compared with a previous
cryo-EM map of the complex. The structure of Osenkowski et al. (18) revealed a somewhat porous structure that was interpreted to have several domains on the extracellular side and solvent-accessible low density cavities in the transmembrane region of the complex. In contrast and despite having comparable dimensions, our structure appears to be more compact, with the extracellular region consisting of a single domain that is consistent with the dimensions of the ectodomain of NCT (65–67). In addition, instead of several low density cavities within the postulated transmembrane region (18), we observed a single central chamber that interfaces with a concave surface on the extracellular region. Although we cannot rule out that these differences could be due to the different resolution of the two maps, an alternative possibility is that they arose due to the different detergents used to solubilize the enzyme. The structure of Osenkowski et al. (18) was determined in the detergent digitonin, under conditions in which, as reported by the authors (55), γ-secretase does not produce measurable physiological ratios of Aβ40 and Aβ42. In contrast, our studies were performed in the detergent CHAPSO, known to promote maximum γ-secretase activity.

The environment and location of the γ-secretase active site have attracted intense attention. Because the cleavage sites on APP C-terminal fragments are located within the hydrophobic region of the membrane, the question of how to include the water needed to carry out the proteolytic reaction has arisen. Sato et al. (68) and Tolia et al. (69) have recently employed substituted cysteine accessibility methods to show that TMD6 and TMD7 of PS1 contribute to the formation of a hydrophilic pore within the membrane. Furthermore, amino acid residues in the luminal portion of TMD6 have been predicted to form a subsite for substrate or inhibitor binding that is exposed to a hydrophilic milieu, whereas those around the GXGD catalytic motif within TMD7 are highly water-accessible (68). In agreement with these biochemical observations, a water-accessible internal cavity can be visualized in our γ-secretase map. The location of this cavity coincides with the approximate location of the γ-secretase active site derived from our labeling experiments with the active site-directed γ-secretase inhibitor B458-C. We hypothesize that this cavity harbors the active site of γ-secretase. Further supporting the notion of a water-accessible active site in γ-secretase, recently solved x-ray structures of rhomboid (70) and site-2 intramembrane proteases (71) exhibit similar features at the catalytic center. In this regard, rhomboid has a water-filled cavity that converges on the catalytic Ser residue and opens to the extracellular side (70), whereas site-2 protease contains a polar channel that allows water entry to the catalytic zinc atom. Other membrane protein enzymes employ a similar strategy, such as Photosystem II (72), in which the Mn₄Ca needed for water oxidation and oxygen generation is located with a supply of coordinated water molecules in an inner hydrophilic cavity that is shielded from the hydrophobic membrane. In these examples, a wall of TMDs is used as a recurrent strategy to create hydrophilic regions that are sequestered from the surrounding membrane lipids (73). The observed cavity in γ-secretase could provide access to the water needed to carry out the hydrolytic intramembrane proteolytic reaction. Aside from providing access to water, we note that the cavity connects with a concave surface in the extracellular region of the complex and with the surrounding lipid environment. A tantalizing possibility is that these connections (Fig. 5C) could constitute the pathways for product release into the extracellular and cytosolic media.

Albeit at moderate resolution, our work provides the first structural glimpse of the trimeric pre-activation intermediate of γ-secretase. The incorporation of PEN-2 into this trimeric complex is essential to trigger endoproteolytic cleavage of PS and for γ-secretase activity (16, 22, 23). Comparison of the EM maps of γ-secretase and its trimeric pre-activation intermediate did not reveal large conformational changes, suggesting that the addition of the smallest subunit, PEN-2, does not cause large structural rearrangements. However, significant differences can be observed in the region attributed to NCT and around the central transmembrane cavity that we have hypothesized to harbor the γ-secretase active site. At the molecular level, endoproteolytic cleavage occurs within a region encoded by the exon 9 peptide that resides between TMD6 and TMD7, which harbors the two catalytic aspartates in PS1. The PS1ΔE9 mutation, in which the exon 9 loop is deleted and therefore lacks the cleavage sites, does not undergo endoproteolysis (13), and yet this mutant is constitutively active (74). Knappenberger et al. (75) have hypothesized that a cleavage-induced conformational change, which relieves the inhibitory effect of the intact exon 9 loop that occupies the substrate-binding site on the immature enzyme, might be responsible for γ-secretase activation. These conformational changes could account for the structural changes observed around the central cavity. As for the changes in the region presumed to correspond to NCT, we note that, using the susceptibility to digestion by trypsin as a measure of conformation, Shirotani et al. (76) have suggested that the incorporation of PEN-2 results in a conformational change in the ectodomain of NCT that leads to γ-secretase activity. This conformational change could be responsible for the observed rearrangements in the extracellular region of the complex. Alternatively, we cannot rule out that these changes upon PEN-2 incorporation are due to the acquisition of complex N-linked oligosaccharide modifications within the NCT ectodomain (26). The work presented here offers some insight into the architecture of γ-secretase and its activation. Ultimately, ongoing efforts to produce higher resolution maps will unravel the mechanism of this fascinating membrane enzyme.

Acknowledgments—We are grateful to David Stokes (New York Structural Biology Center) for support and help throughout this work and the New York Structural Biology Center for access to state-of-the-art electron microscopy facilities and excellent staff.

REFERENCES
1. Sloane, P. D., Zimmerman, S., Suchindran, C., Reed, P., Wang, L., Boustani, M., and Sudha, S. (2002) Annu. Rev. Public Health 23, 213–231
2. Price, D. L., Sisodia, S. S., and Borchelt, D. R. (1998) Science 282,
26. Tolia, A., Chávez-Gutiérrez, L., and De Strooper, B. (2006) J. Biol. Chem. 281, 27633–27642
69. Wang, Y., Zhang, Y., and Ha, Y. (2006) Nature 444, 179–180
70. Feng, L., Yan, H., Wu, Z., Yan, N., Wang, Z., Jeffrey, P. D., and Shi, Y. (2007) Science 318, 1608–1612
71. Kern, J., Biesiadka, J., Loll, B., Saenger, W., and Zouni, A. (2007) Photosynth. Res. 92, 389–405
72. Steiner, H., Romig, H., Grim, M. G., Philipp, U., Pesold, B., Citron, M., Baumeister, R., and Haass, C. (1999) J. Biol. Chem. 274, 7615–7618
73. Knappenberger, K. S., Tian, G., Ye, X., Sobotka-Briner, C., Ghanekar, S. V., Greenberg, B. D., and Scott, C. W. (2004) Biochemistry 43, 6208–6218
74. Shirotani, K., Edbauer, D., Capell, A., Schmitz, J., Steiner, H., and Haass, C. (2003) J. Biol. Chem. 278, 16474–16477