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Generation of Monoclonal Antibody Fragments Binding the Native γ-Secretase Complex for Use in Structural Studies

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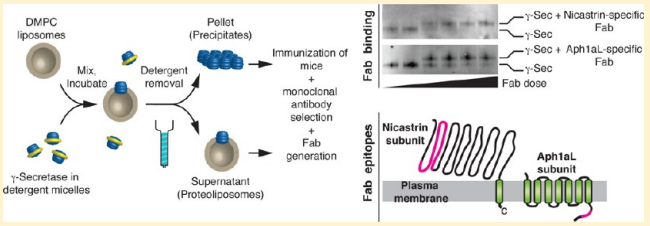
Supporting Information

ABSTRACT: A detailed understanding of γ-secretase structure is crucially needed to elucidate its unique properties of intramembrane protein cleavage and to design therapeutic compounds for the safe treatment of Alzheimer's disease. γ-Secretase is an enzyme complex composed of four membrane proteins, and the scarcity of its supply associated with the challenges of crystallizing membrane proteins is a major hurdle for the determination of its high-resolution structure. This study addresses some of these issues, first by adapting CHO cells overexpressing γ-secretase to growth in suspension, thus yielding multiliter cultures and milligram quantities of highly purified, active γ-secretase. Next, the amounts of γ-secretase were sufficient for immunization of mice and allowed generation of Nicastrin- and Aph-1-specific monoclonal antibodies, from which Fab fragments were proteolytically prepared and subsequently purified. The amounts of γ-secretase produced are compatible with robot-assisted crystallogenesis using nanoliter technologies. In addition, our Fab fragments bind exposed regions of native γ-secretase in a dose-dependent manner without interfering with its catalytic properties and can therefore be used as specific tools to facilitate crystal formation.



γ-Secretase is a high-molecular mass protein complex composed of four integral membrane proteins, Presenilin (PS), Nicastrin (NCT), anterior pharynx defective 1 (Aph1), and Presenilin enhancer 2 (Pen2), with PS constituting the catalytic component and the other three subunits acting as necessary cofactors (Figure 1a[1]). This aspartyl protease is the founding member of an emerging class of intramembrane-cleaving proteases, including site 2 protease (S2P), rhomboids, and signal peptide peptidase (SPP), all of which hydrolyze their respective substrates within the transmembrane regions.[2]

From functional and pharmacological points of view, γ-secretase is involved in regulated intramembrane proteolysis of a growing list of single-pass type I transmembrane proteins, including, for instance, the amyloid precursor protein (APP), the Notch receptor, the cell adhesion molecule N-Cadherin, and the more recently identified Neurexin synaptic protein.[3,4] Because γ-secretase is the enzyme responsible for the final step in production of amyloid-β peptides (Aβ), the key causative agents of Alzheimer's disease, considerable effort has been spent on the development of therapeutic γ-secretase inhibitors. However, recent phase 3 clinical trials with such compounds revealed adverse side effects resulting from inhibition of Notch cleavage, a transmembrane receptor involved in regulation of cell fate decisions.[5] Selective γ-secretase modulators are thus actively sought to lower the level of Aβ production, while preserving vital activities of the protease.[6,7]

X-ray crystallography is a well-established approach to understanding substrate recognition and enzyme mechanisms[8−10] and has a substantial track record in rational drug design on the basis of three-dimensional structure information.[11] Therefore, not only can the detailed knowledge of γ-secretase structure help elucidate the mechanism of intramembrane proteolysis, it will undoubtedly advance the design and development of drugs for the safe treatment of Alzheimer's disease, as well as several types of cancer.

Limited amounts of purified γ-secretase have so far allowed imaging of single particles by electron microscopy (EM) and cryo-EM and provided low-resolution structural insight into the γ-secretase internal cavity and 20 Å pores located at either end.[1,12,13] However, further progress toward determining a high-resolution structure by X-ray or two-dimensional crystallography has been limited by many challenges in overexpression, purification, and functional characterization of active γ-secretase particles in amounts far greater than those typically used for biochemical or imaging studies. The crystallization process of transmembrane proteins like γ-secretase is itself highly challenging and is often facilitated by or even requires additional reagents like specific Fab fragments of monoclonal antibodies to promote crystal contacts as seen in recent breakthroughs with other transmembrane systems.[14−17]

To advance the determination of a high-resolution structure of γ-secretase, we report here a procedure aimed at producing

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the protease complex in high yield and purity, as well as generating specific antibody tools to facilitate the crystallogenesis. The experiments include adapting a CHO cell line expressing active γ-secretase to growth in suspension, preparation of γ-secretase under conditions that are suitable for crystallization experiments and immunization of mice, screening of γ-secretase-specific monoclonal antibodies, and Fab fragment preparation and characterization.

MATERIALS AND METHODS

Materials. *N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-(*S*)-phenylglycine-*tert*-butyl ester (DAPT), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), egg yolk 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC), and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE) were purchased from Sigma-Aldrich (Steinheim, Germany). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, G418 sulfate, zeocin, hygromycin, and blasticidin were purchased from Invitrogen (Carlsbad, CA). Puromycin was purchased from VWR (Randor, PA). Gel filtration calibration standards blue dextran 2000, thyroglobulin, and ferritin were purchased from GE Healthcare (Wauwatosa, WI).

Cell Lines and Cultures in Suspension. The optimization of γ-secretase expression in suspension cultures was performed with CHO-based S20 cells, which stably overexpress APP751, human Aph1aL-HA, Flag-Pen2, Presenilin-1 (PS1), and Nicastrin-v5 (NCT-v5).[18] Under standard conditions, adherent S20 cells were grown on plates in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, 150 μg/mL G418, 25 μg/mL puromycin, 250 μg/mL zeocin, 250 μg/mL hygromycin, and 10 μg/mL blasticidin. For small-scale (<50 mL) cultures in suspension, cells were inoculated at a density of 0.5×10^6 cells/mL in 5 mL of ProCHO5 prewarmed medium (Lonza Verviers, Verviers, Belgium) containing 1% FBS in CultiFlask 50 tubes (Sartorius AG, Göttingen, Germany). The cultures were agitated for 3−4 days by orbital shaking at 180 rpm in an ISF-4-W incubator (Kühner AG, Birsfelden, Switzerland) at 37 °C in the presence of 5% CO_2.[19] Cells were counted, diluted to a density of 0.5×10^6 cells/mL with medium, and transferred as 5−10 mL aliquots into CultiFlask 50 tubes. The cultures were incubated with orbital shaking as described above. In the case of 1 L cultures, cells were first inoculated in 100 mL of ProCHO5 with 1% FBS medium at a density of 0.5×10^6 cells/mL in a 250 mL square-shaped glass bottle (Schott Glass, Mainz, Germany) with the bottle cap opened by one-quarter of a turn. The culture was agitated as described above by orbital shaking at 110 rpm and 37 °C in the presence of 5% CO_2 for 2−3 days.[19] Cells were counted and diluted to a density of 0.5×10^6 cells/mL in a volume of 400 mL in a 1 L square-shaped glass bottle. After 2−3 days, the culture was diluted to 1 L in a 5 L glass bottle and incubated on a shaker for 3 days as described above. In the case of cultures exceeding 1 L, the cells were diluted with fresh medium. At the final 10 L stage, the culture was divided into six 5 L cylindrical glass bottles (1.7 L per bottle) and agitated at 110 rpm and 37 °C as described above. After 2−3 days, cells reached a density of $1.8–2.0 \times 10^6$ cells/mL and were harvested by centrifugation at 350*g* for 5 min. Cell pellets were washed once with PBS and stored at −80 °C.

Human (HeLa and HEK293T) or rodent (CHO and MEF) cell lines were grown in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Preparation of Microsomal Extracts. Microsomal preparations for γ-secretase activity assays and Western blot analysis were performed as previously described.[18] Briefly, cells were harvested, and total membranes were prepared by differential centrifugation of the lysate to remove cytosolic proteins, followed by a bicarbonate wash to remove peripheral membrane proteins. Microsomal extracts were obtained by solubilization in buffer [50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$] containing 1% (w/v) CHAPSO, followed by centrifugation (15700*g*) to remove the insoluble material.

γ-Secretase Activity Assays. In vitro γ-secretase assays using the recombinant APP-based C100-Flag were performed as previously reported.[18,20,21] Briefly, purified γ-secretase or S20 whole cell or microsomal extracts were dissolved in 0.2 or 0.25% (w/v) CHAPSO, respectively, 50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$ and incubated at 37 °C for 4 h with 1 μM substrate, 0.1% (w/v) PC, and 0.025% (w/v) PE. The reactions were stopped by adding Laemmli sample buffer for Western blot analysis. Resulting products, APP intracellular domain (AICD)-Flag and Aβ, were detected with specific antibodies as indicated or analyzed by mass spectrometry.

Western Blotting and Antibodies. Whole cell and microsomal extracts from S20 cells were prepared by solubilization in 50 mM HEPES buffer (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$, containing indicated detergent concentrations. Samples for Western blot analysis of γ-secretase components were run on 12% Tris-glycine sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels, transferred onto nitrocellulose membranes, and probed with NCT164 (NCT-specific, 1:1000, BD Biosciences, Franklin Lakes, NJ), MAB1563 (PS1-NTF-specific, 1:1000, Merck Millipore, Billerica, MA), anti-PS1 (1:1000, Ab10281, Abcam, Cambridge, U.K.), UD-1 (Pen2-specific, 1:500, gift from H. Karlström), M2 (Flag-specific, 1:1000, Sigma-Aldrich), anti-actin (1:2000, A2066, Sigma-Aldrich), O2C2 (Aph1aL-specific, 1:1000, Covance, Princeton, NJ), or 3F10 (HA-specific, 1:2000, Roche Applied Science, Penzberg, Germany) antibodies.

Native samples were run on 4 to 16% BN-PAGE gels (Invitrogen) according to the manufacturer's instructions, and γ-secretase was revealed with NCT-specific NCT164 (Figure 2a and Figure S2e of the Supporting Information), with our hybridoma supernatants [1:100 (Figure S2e of the Supporting Information)], or with PS1-specific antibodies MAB1563 and anti-PS1 (Figure 4b) using the dilutions mentioned above.

Samples from cell-free γ-secretase activity assays were run on 4 to 12% Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes to detect Aβ, APP intracellular domain (AICD)-Flag with Aβ-specific 6E10 antibody (1:1000) (Covance, Berkeley, CA), and Flag-specific M2 antibody.

γ-Secretase Immunoprecipitation. Native or denatured protein extracts were prepared by solubilizing CHO cells overexpressing γ-secretase in 1% (w/v) CHAPSO or NP-40, respectively, and 50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. 2D5, 2G6, 2F12, and 2H10 antibodies from hybridoma supernatants were purified by caprylic acid precipitation and mixed (1:200 final dilution) with 50 μL of Protein G agarose (Roche Applied Science) and

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protein extracts. Start, unbound, and IP fractions were analyzed by SDS−PAGE and immunoblotted with NCT164, anti-PS1, 3F10, and M2 antibodies to detect subunits NCT, PS1, Aph1-HA, and Flag-Pen2, respectively.

Gel Filtration Chromatography. Gel filtration chromatography of the γ-secretase complex was performed on a Superdex 200 10/300 GL column (GE Healthcare); 150 μL of 20-fold concentrated γ-secretase was loaded and eluted with 0.1% digitonin-TBS at a rate of 0.3 mL/min. The column was calibrated with soluble standards blue dextran 2000 (void volume), thyroglobulin (669 kDa), and ferritin (440 kDa).

Liposome Preparation. Thin lipid films were formed by evaporating chloroform from DMPC solutions under a stream of N_2. The dried films were then rehydrated in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl and then sonicated in a bath sonicator at 65 °C until the solution had become clear.

Antigen Preparation for Immunization. γ-Secretase (0.3 mg) was purified from 3 L of S20 suspension cultures by following the protocol described previously[18] and concentrated 15-fold on filter concentrators with a molecular mass cutoff of 30000 Da. Proteoliposomes were prepared by incubating DMPC liposomes with concentrated γ-secretase containing 0.1% (w/v) digitonin-TBS for 1 h at 4 °C. Buffer exchange was then performed by gel filtration on G-25 minitrap columns (GE Healthcare) to remove digitonin from the mix. The eluate was centrifuged at 15000*g*, and Western blots showed an equal presence of γ-secretase in the supernatant and pellet (Figure 2d). Both fractions were therefore used in separate immunizations.

Immunization of Mice with γ-Secretase and Antibody Generation. Supernatant and pellet fractions of γ-secretase reconstituted in phospholipid were used for separate immunizations. Supernatant or pellet γ-secretase (50 μg) supplemented with 200 μg of polyinosinic-polycytidylic acid (poly I/C) adjuvant was injected three times intraperitoneally into 8-week-old female BALB/c mice (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France) at 14-day intervals. Mice with positively reacting sera were reinjected 4 days before hybridoma fusion of spleen cells with Sp2/0 Agl4 myeloma cells as described previously.[22] Hybridoma supernatants were assayed 10 days later by an enzyme-linked immunosorbent assay (ELISA), dot blot, and cell imaging for cross reactivity with γ-secretase (Figure S2d of the Supporting Information). Specific cultures were cloned twice, and specific hybridomas were established. The antibodies were found to be of subclass IgG using an isotyping kit (Roche Applied Science). Monoclonal anti-γ-secretase antibodies selected for further experiments were purified using the caprylic acid method.[23] All animal procedures were performed in accordance with European and ARRIVE guidelines. The permission to experiment on animals was delivered by the departmental direction of veterinary services under number 67-351 (Strasbourg, France).

ELISAs. For screening anti-γ-secretase antibodies (Figure S2d of the Supporting Information), a microsomal preparation of S20 cells expressing γ-secretase was diluted in 0.2% (w/v) CHAPSO-PBS and incubated at 4 °C overnight in ELISA plates. After being coated, the plates were washed with 0.05% (w/v) Tween in PBS, and nonspecific binding was blocked by 1% (w/v) gelatin in PBS over 30 min at room temperature. The plates were washed, and 100 μL of each hybridoma supernatant was incubated in plate wells for 1 h at room temperature. Washing steps were repeated, and anti-mouse secondary antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch laboratories, West Grove, PA) was incubated for 1 h. Following two washing steps, binding of the antibody to γ-secretase was revealed with 1 mg/mL *p*-nitrophenyl phosphate, 1 M diethanolamine (pH 9.8), and 0.5 mM $MgCl_2$. Absorbances were measured at 405 nm on a Multiskan MS microplate reader (Thermo Labsystems, Helsinki, Finland). For epitope mapping of Aph1-specific monoclonal antibodies (Figure 3e), the plates were incubated with the indicated peptide solutions. Control antibodies O2C2 (Aph1aL-specific, Covance) and 12CA5 (HA-specific, produced in house by IGBMC) were included in this assay.

Live Cell Staining. S20 or untransfected CHO cells were grown in 96-well plates and incubated for 15 min at 37 °C and 5% CO_2 with either hybridoma supernatants (Figure S2b,d of the Supporting Information) or purified antibodies (Figure 3b) diluted 1:4 or 1:200, respectively. The plates were washed twice with DMEM containing 10% FBS, incubated with donkey anti-mouse secondary antibody coupled to fluorescent Cy3 dye (Jackson ImmunoResearch laboratories; 1:400 dilution in DMEM containing 10% FBS) for 15 min at 37 °C and 5% CO_2, and finally washed three times with DMEM containing 10% FBS. The plates were then washed twice with cold (∼4 °C) PBS before fluorescence measurements were taken using a TECAN Safire2 microplate reader (TECAN Group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 553 and 576 nm, respectively. Cells were imaged on an inverted Leica DMI4000 epifluorescence microscope (Leica microsystems, Wetzlar, Germany).

Fixed Cell Staining. S20 or untransfected CHO cells were grown in 96-well plates, washed with PBS, fixed with 4% (w/v) paraformaldehyde for 15 min, and washed again three times with PBS. The fixed cells were treated with 33 mM NH_4Cl for 10 min, washed twice with PBS, permeabilized with 0.25% (w/v) Triton X-100 for 5 min, and finally blocked with 0.25% (w/v) gelatin (G7765, Sigma-Aldrich) in PBS for 50 min. Each well was incubated with either hybridoma supernatants (Figure S2c,d of the Supporting Information) or purified antibodies (Figure 3b) diluted 1:6 or 1:200, respectively. All dilutions were made in 90 μL of PBS containing 0.125% (w/v) gelatin for 90 min at 37 °C or overnight at 4 °C. The plates were washed three times with PBS containing 0.125% (w/v) gelatin, incubated with secondary antibody [donkey anti-mouse Cy3 (Jackson ImmunoResearch laboratories), 1:400 in PBS containing 0.125% (w/v) gelatin] for 1 h at room temperature, and then washed three times with PBS containing 0.125% (w/v) gelatin. Fluorescence was measured, and cells were imaged as described above.

Generation of Fab Fragments. Purified monoclonal antibodies were subjected to enzyme digestion by immobilized ficin in the presence of 25 mM cysteine and purified on Protein A resin according to the instructions of the Pierce mouse IgG1 Fab and $F(ab')_2$ preparation kit (Thermo Fisher Scientific, Waltham, MA). The purified digests were evaluated by Coomassie-stained SDS−PAGE under reducing conditions.

Immunoprecipitation−Mass Spectrometry (IP−MS) Analysis of Aβ. Aβ generated in γ-secretase in vitro assays was analyzed as previously described.[21] Briefly, Aβ was IPed overnight using monoclonal anti-Aβ antibody 4G8 (Covance, Berkeley, CA) and protein G coupled to agarose resin (Roche Applied Science) and then recovered by elution with a 1:1 (v/v) acetonitrile/H_2O mixture containing 0.1% (v/v) trifluoroacetic acid. Eluted fractions were equally mixed with saturated CHCA (α-cyano-4-hydroxycinnaminic acid). The fractions

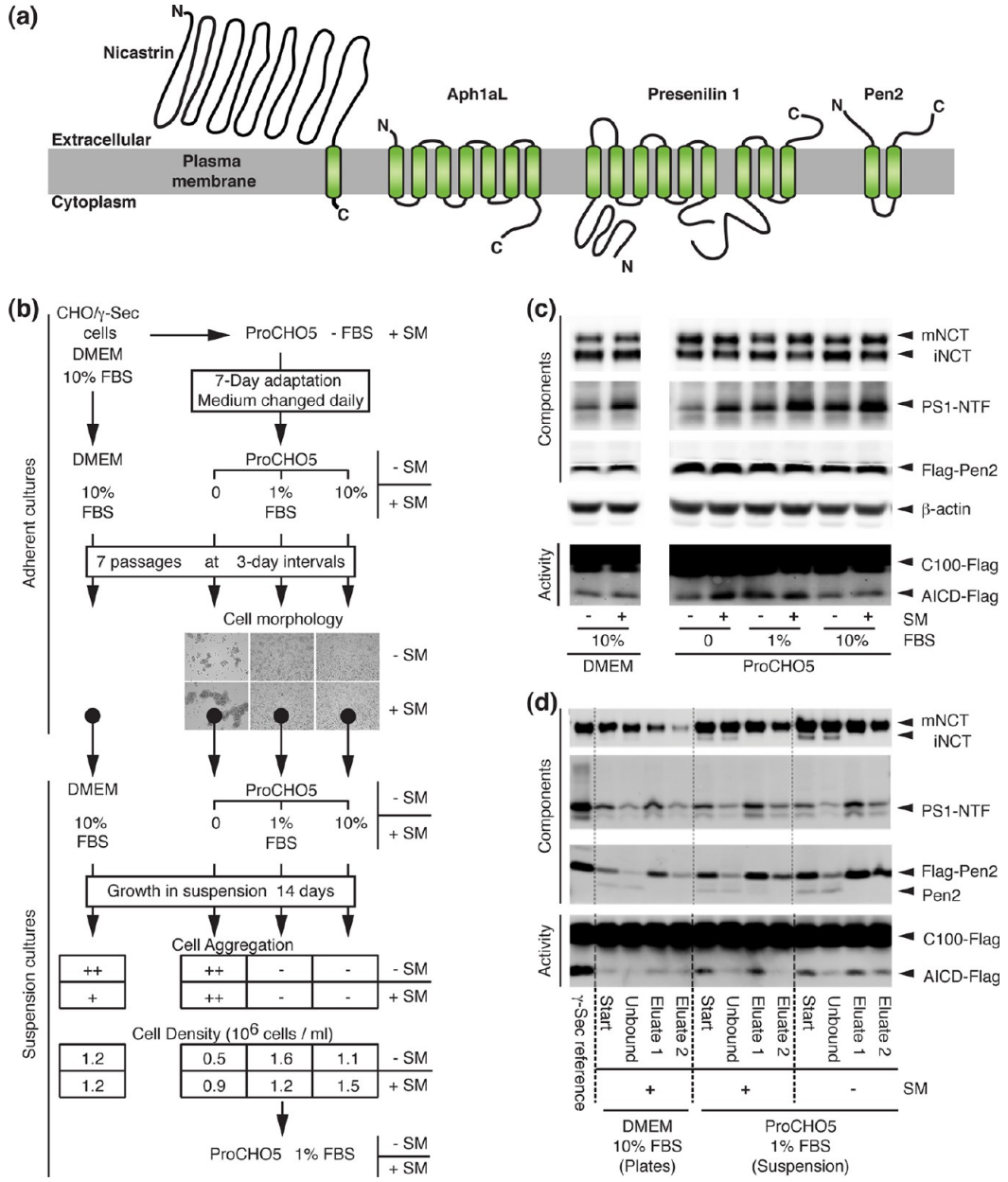

Figure 1. Optimization of suspension growth conditions for large-scale production in bioreactors of CHO cells overexpressing γ-secretase. (a) Topology of the transmembrane protein subunits in the γ-secretase complex. (b) Optimization scheme. CHO cells stably overexpressing γ-secretase (S20 cells[18]) were initially plated in DMEM medium with 10% FBS and selection markers (SM) and then adapted to ProCHO5 medium containing SM and no FBS over 7 days. Adherent cell culturing in ProCHO5 with 0, 1, and 10% FBS with (+) or without (−) SM was then performed in plates, together with DMEM controls with or without SM. Cell morphologies after seven passages are shown (for enlarged versions of the images, see Figure S1 of the Supporting Information). Adherent cells cultured with SM were used to start suspension cultures in the respective media with or without SM. After 14 days, ProCHO5 cultures with 1 or 10% FBS remained aggregation-free and the cell density was highest for medium with 1% FBS without SM. Cell aggregation was estimated qualitatively by inspection under the microscope, and three states were arbitrarily defined: high levels of aggregates (++), medium or low levels of aggregates (+), and no aggregates (−). Cell densities were estimated from a single set of experiments by using the packed cell volume (PCV) approach, as previously described.[44] (c) γ-Secretase expression levels and activity for S20 suspension cultures. Total protein extracts from cells grown for 14 days in suspension (b) were solubilized in 1% CHAPSO, analyzed by Western blotting for γ-secretase components (top), or used to perform γ-secretase cleavage assays (5 μg of protein/assay). AICD cleavage products were revealed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting (bottom). β-Actin was used as a loading control. (d) Medium-scale purification of γ-secretase from cells grown in suspension. S20 cells were grown in suspension for 10 days in 1 L of a ProCHO5/1% FBS mixture with or without SMs, or as adherent cultures for 7 days on plates containing DMEM, 10% FBS, and SM. Affinity purification of γ-secretase from approximately 10^9 cells under each growth condition was performed using Flag-specific M2 agarose and eluted twice with Flag peptides. Prepurification (start), unbound fractions, and first and second eluates were analyzed by SDS−PAGE and Western blotting for γ-secretase subunits. The purified fractions were assayed for γ-secretase activity and AICD cleavage products revealed as described above. γ-Secretase subunits Nicastrin (mature, mNCT; immature, iNCT) and the Presenilin-1 N-terminal fragment (PS1-NTF) (c and d) were detected using antibodies NCT164 and MAB1563, respectively. Flag-tagged Pen2 (c) was detected with antibody M2. Endogenous and Flag-tagged Pen2 (d) were detected using antibody UD-1. APP cleavage product AICD-Flag was detected with the M2 antibody (c and d).

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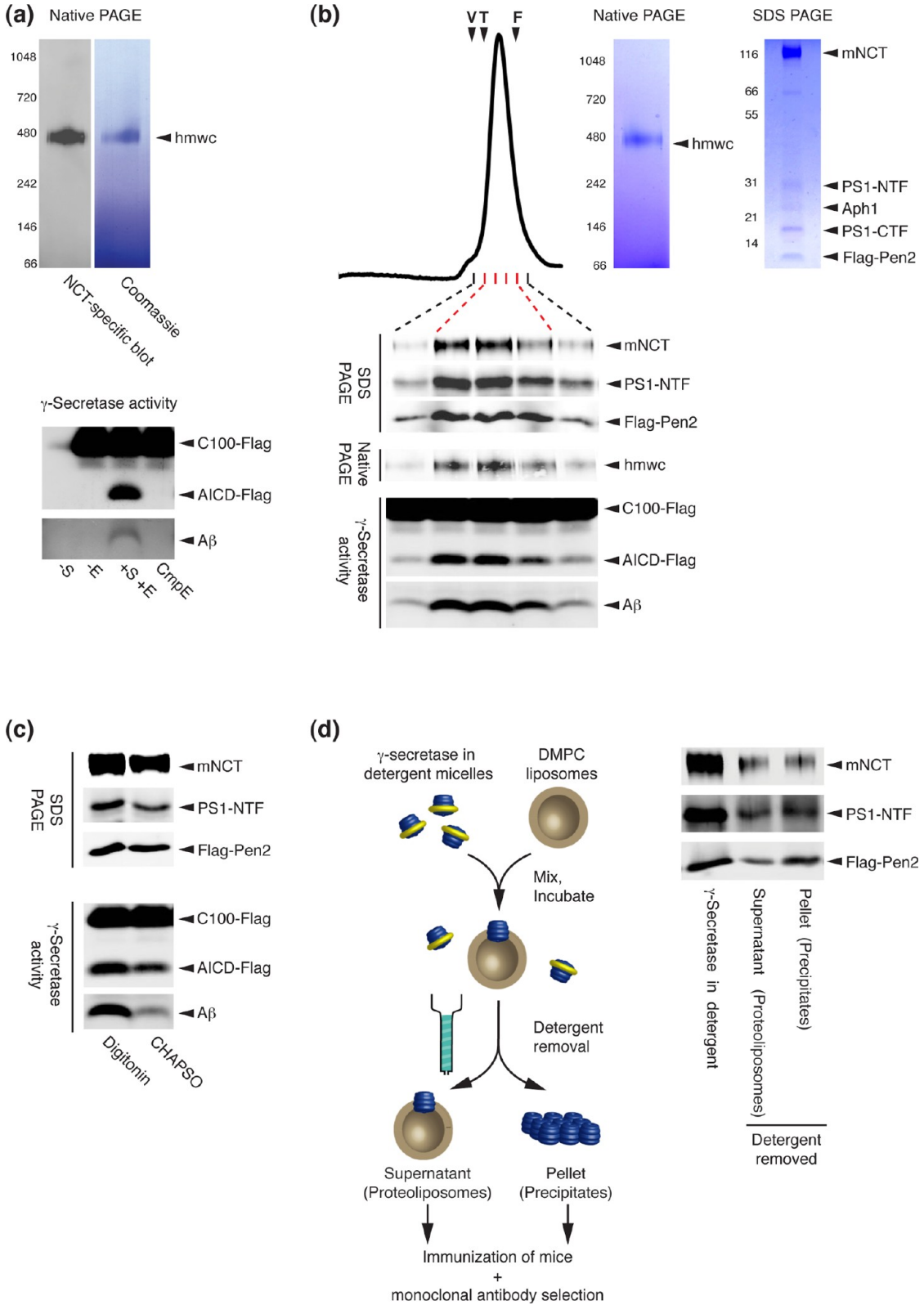

Figure 2. Large-scale preparation of purified γ-secretase and its reconstitution in lipid membranes for immunization of mice. (a) γ-Secretase solubilized in 0.1% (w/v) digitonin-TBS was purified from S20 cells produced in large-scale suspension cultures. The purity, homogeneity, and integrity of the γ-secretase high-molecular mass complex (hmwc) were assessed by Coomassie-stained BN-PAGE and Western blotting with NCT-specific antibody NCT164 (top). Activity assays were performed with purified γ-secretase, including control reactions with omitted substrate (−S) or γ-secretase (−E), or with added γ-secretase inhibitor Compound E (CmpE, 10 μM). Assay reactions were analyzed by SDS−PAGE, and APP cleavage products AICD-Flag and Aβ were detected with M2 and 6E10 antibodies, respectively (bottom). (b) Purified γ-secretase was concentrated, loaded on a Superdex 200 gel filtration column, and eluted with 0.1% (w/v) digitonin-TBS. In the elution profile, positions of the void volume and elution volumes for molecular mass standards thyroglobulin (669 kDa) and ferritin (440 kDa) are indicated by V, T, and F, respectively. Elution peak fractions (delimited by black tick marks) were analyzed by SDS−PAGE and Native-PAGE and immunoblotted with NCT-specific antibody (SDS−

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Figure 2. continued

PAGE and Native-PAGE) and PS1-NTF- and Flag-specific antibodies (SDS−PAGE). The fractions were also assayed for γ-secretase activity as described above. Three fractions showing the largest amounts of γ-secretase and highest activities (indicated by red tick marks) were pooled, concentrated, and analyzed by Coomassie-stained Native-PAGE and SDS−PAGE (top middle and right, respectively). (c) γ-Secretase was prepared in 0.5% CHAPSO-TBS and compared to the protein complex prepared in 0.1% digitonin-TBS. Both preparations were analyzed by SDS−PAGE and Western blotting to detect γ-secretase components NCT, PS1-NTF, and Pen2 and assayed for enzyme activity. (d) γ-Secretase reconstitution in lipid membranes for immunization of mice. In the scheme at the left, purified γ-secretase was incubated with DMPC liposomes for 1 h at 4 °C. Digitonin was removed from the mix by gel filtration, after which the eluate was centrifuged at 15000*g*. Both the supernatant and the pellet were analyzed by SDS−PAGE and Western blotting to detect γ-secretase subunits NCT, PS1-NTF, and Flag-Pen2 using antibodies NCT164, MAB1563, and M2, respectively (right). Both fractions were therefore used in separate immunizations.

were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry in reflectron mode on an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Carlsbad, CA). Molecular masses were accurately measured and searched against amino acid sequences of human APP-C99 with addition of a methionine residue at the N-terminus and a Flag tag sequence at the C-terminus (C100-Flag).

RESULTS

Development of Large-Scale Suspension Cultures of CHO Cells Overexpressing γ-Secretase. Ideal cellular systems for γ-secretase overexpression have to fulfill the following criteria. First, assembly and maturation of the enzymatic complex should be accompanied by highly regulated endoproteolytic processing of Presenilin-1 to generate N- and C-terminal fragments PS1-NTF and PS1-CTF, respectively (reviewed in refs 24 and 25), which is associated with the biological activity of γ-secretase.[26,27] Next, human glycosylation types should be preserved because NCT is a type I membrane protein present in active γ-secretase as a "mature" form, which is characterized by N-linked glycosylations. These result from post-translational modification following the entry of NCT into the endoplasmic reticulum and the Golgi apparatus.[28−30] Finally, large numbers of harvested cells should be obtained at affordable cost and in reasonable time to achieve milligram amounts of highly purified protein for structure determination.

Accordingly, we opted for the CHO-based S20 cell line, which fulfills those requirements,[18] and we proceeded to adapt the cells to chemically defined media designed for cell growth in suspension. The adaptation was critical because S20 cells were initially developed for adherent growth in culture dishes, which could be influenced by several parameters, including basal medium, fetal bovine serum (FBS) concentration,[31] and the presence of selection markers (SM). The adaptation was performed near confluency in a 10 cm dish containing adherent S20 cells by replacing the medium (DMEM, 10% FBS, and SM) daily for 7 days with ProCHO5 in the absence of FBS. SM were added to maintain selection pressure throughout the adaptation process (Figure 1b).

Following this initial adaptation stage, we tested the effect of FBS and SM on cell morphology, adherence, expression of γ-secretase subunits, and activity. For that purpose, cells were grown in ProCHO5 at 0, 1, or 10% FBS in the presence or absence of SM, and these were compared with cells grown in regular DMEM containing 10% FBS, also in the presence or absence of SM (Figure 1b). All cells were maintained at high confluency for 21 days with regular passages at 3 day intervals. As shown in Figure 1b and Figure S1 of the Supporting Information, the cell morphology and adherence in ProCHO5 depended exclusively on serum concentration. In the absence of FBS, the cells did not adhere to the culture plate and instead formed suspended clumps, while at 1% FBS, approximately half the population was adherent. In contrast, the cells remained nearly entirely attached at 10% FBS (Figure 1b and Figure S1 of the Supporting Information).

In the second stage of development, the transition from adherent to suspension cultures was performed on a small scale to monitor cell properties and γ-secretase expression. To avoid the potential loss of γ-secretase expression, starter cells were exclusively taken from cells grown in the presence of SM. Adherent S20 cells adapted in ProCHO5 with 0, 1, or 10% FBS, as well as control S20 cells previously maintained in standard DMEM with 10% FBS, were detached and grown in suspension for 14 days, in the presence or absence of SM. Cell aggregation was monitored because it is a determining factor of cell performance and density in suspension cultures. Assessment by optical microscopy revealed that ProCHO5 cultures containing 1 or 10% FBS were exclusively aggregation-free. This was in sharp contrast to FBS-free ProCHO5 cultures, characterized by high levels of cell aggregation associated with low cell densities. Indeed, cell densities were consistently high under all conditions that included FBS, regardless of any other parameters (Figure 1b). At the end of this culture period, γ-secretase components in total cell extracts were analyzed by Western blot, and the activity of γ-secretase in microsomal preparations was estimated as previously described.[18] As shown in Figure 1c, the optimal medium for γ-secretase expression in S20 suspension cultures appeared to be ProCHO5 supplemented with 1% FBS, as this combination yielded the highest protein and activity levels with minimal cell aggregation.

In the final stage, we assessed the yield of active γ-secretase purified from medium-scale cultures of S20 cells in suspension. We used the optimal medium conditions determined in the previous stages (ProCHO5 with 1% FBS) and compared the yield with that of standard adherent cultures (DMEM with 10% FBS). Because the majority of cell production cost is due to SM, we also examined at this stage the effect of SM on the yield, purity, and activity of the final γ-secretase preparations. Two suspension cultures of S20 cells were started in 100 mL of ProCHO5 containing 1% FBS and SM until a density of 0.5×10^6 cells/mL was reached and then expanded in 1 L of ProCHO5 with 1% FBS (with SM maintained in only one culture). S20 cells were grown until a density of 2×10^6 cells/mL was reached, and then approximately 10^9 cells were harvested from each suspension culture and from the standard plate culture. Next, γ-secretase was affinity purified using the Flag tag N-terminally fused to the Pen2 subunit according to a protocol described previously.[18] As expected, we found only mature NCT in purified γ-secretase fractions and no endogenous Pen2 (Figure 1d). Most importantly, no major differences in γ-secretase yields and activities were observed

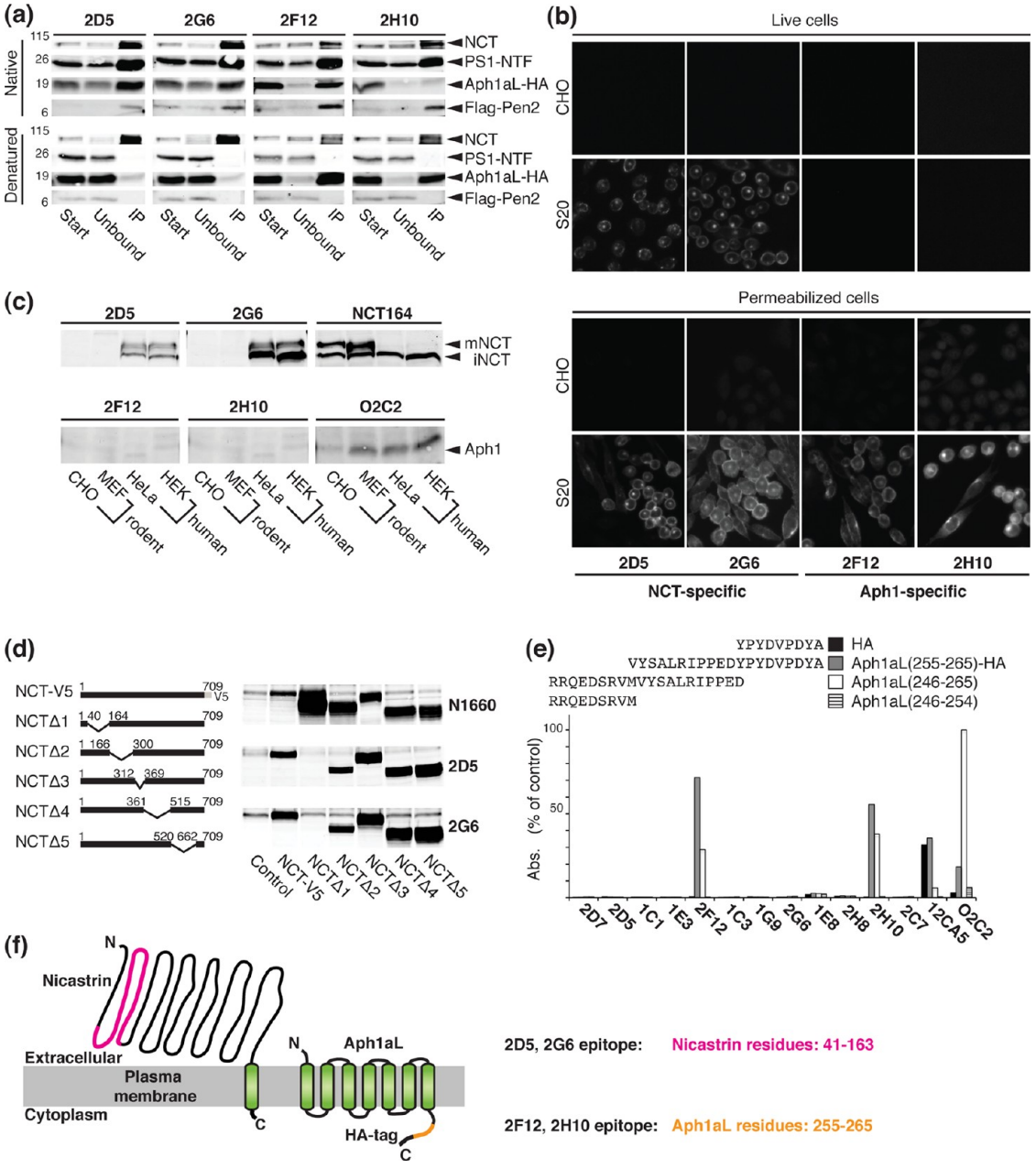

Figure 3. Characterization and epitope mapping of selected γ-secretase monoclonal antibodies (mAbs). (a) γ-Secretase immunoprecipitation (IP) with selected mAbs. Native or denatured protein extracts were prepared by solubilizing S20 cells in 1% (w/v) CHAPSO-containing or 1% (w/v) NP-40-containing buffers, respectively. mAbs 2D5, 2G6, 2F12, and 2H10 purified by caprylic acid from hybridoma supernatants were mixed (final dilution of 1:200) with Protein G agarose and protein extracts. Start, unbound, and IP fractions were analyzed by SDS−PAGE and immunoblotted with antibodies NCT164, anti-PS1, 3F10, and M2 to detect subunits NCT, Presenilin, Aph1-HA, and Flag-Pen2, respectively. (b) Cell immunostaining with selected γ-secretase-specific mAbs. S20 and control CHO cells were incubated with purified mAbs 2D5, 2G6, 2F12, and 2H10, either under live conditions or after paraformaldehyde fixation and permeabilization with Triton X-100, and further incubated with a mouse-specific secondary antibody coupled to the Cy3 dye. Note that γ-secretase in live cells is detected by only NCT-targeting antibodies. (c) Immunoreactivity of selected mAbs to endogenous γ-secretase complexes. Denatured protein extracts from rodent (CHO and MEF) or human (HeLa and HEK293T) cell lines solubilized in 1% (w/v) NP-40-containing buffer were subjected to SDS−PAGE and immunoblotted with purified NCT-specific (2D5 and 2G6) or Aph1-specific (2F12 and 2H10) mAbs. Control blots were performed with antibodies NCT164 (NCT-specific) or O2C2 (Aph1-specific). (d) Epitope mapping of NCT-specific mAbs. HEK293T cells were transfected with several deletion mutants of human NCT[35] (left), and 1% NP-40 whole cell extracts were analyzed by SDS−PAGE and immunoblotting with the 2D5, 2G6, or N1660 mAb specific for the C-terminus of NCT (right). An extract from untransfected cells was included as a control. (e) Epitope mapping of Aph1-specific mAbs. Binding of an antibody to four peptides encompassing the C-terminal amino acid sequence of HA-tagged Aph1aL was measured with an ELISA. Commercial antibodies O2C2 and 12CA5, specific for the C-terminus of the Aph1 and the HA tag, respectively, were included as controls. Peptide sequences, including the start and end positions within Aph1aL, are indicated. (f) Epitope localization of selected mAbs in the topology of γ-secretase subunits NCT (purple, antibodies 2D5 and 2G6) and Aph1aL-HA (orange, antibodies 2F12 and 2H10). The epitopes are located on opposite sides of the plasma membrane.

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between either suspension culture [with or without SM (Figure 1d)], both of which having outperformed the adherent culture. We concluded that SM-free suspension cultures of S20 cells in ProCHO5 supplemented with 1% FBS were the optimal condition for large-scale and low-cost production of purified γ-secretase.

Large-Scale Preparation of Purified γ-Secretase. By combining the culture conditions described above with a purification protocol described previously,[18] we prepared approximately 0.8 mg of γ-secretase from 10 L of S20 suspension culture, as estimated by three different methods (Figure S3 and Table S1 of the Supporting Information). Blue Native-PAGE (BN-PAGE) of the concentrated γ-secretase preparation showed a unique band, which can be detected by both immunoblotting and Coomassie staining (Figure 2a, top), confirming the high-grade purity of our γ-secretase preparation. In addition, an activity assay performed with this preparation and APP-based recombinant substrate C100-Flag confirmed the enzyme was active and had a typical response to specific inhibitors (Figure 2a, bottom).

High-resolution structure determination methods like protein crystallography require the protein sample to be stable and monodisperse. We assessed these properties in the γ-secretase preparation by gel filtration chromatography (Figure 2b). The elution profile shows a single, well-resolved peak positioned between elution volumes of molecular mass markers ferritin (440 kDa) and thyroglobulin (669 kDa). Western blot analysis of peak fractions confirms the presence of γ-secretase components mNCT, PS1-NTF, and Pen2 (Figure 2b, bottom, SDS−PAGE), as well as native γ-secretase (Figure 2b, bottom, Native-PAGE). γ-Secretase activity is preserved in eluted peak fractions as seen from the production of cleavage products AICD-Flag and Aβ (Figure 2b, bottom). Peak fractions were pooled, concentrated, and further analyzed by Native-PAGE and SDS−PAGE. A single band was detected in Coomassie-stained Native-PAGE (Figure 2b, top), which is very similar to the single band detected prior to gel filtration (Figure 2a, top). Coomassie-stained SDS−PAGE shows distinct bands corresponding to the mature components of the γ-secretase complex [mNCT, PS1-NTF, Aph1, PS1-CTF, and Pen2 (Figure 2b, top)].

Although there are reports of membrane protein preparations in digitonin followed by successful crystallization,[32,33] we decided to investigate the properties of γ-secretase prepared in CHAPSO, because this detergent has been used in the crystallization of GPCR and ion channel membrane proteins.[15,32,33] We found that γ-secretase is easily prepared and purified when 0.5% CHAPSO is used instead of 0.1% digitonin in the regular protocol. Although the purification yield in CHAPSO is slightly lower (Figure 2c), the complex integrity is confirmed by the presence of subunits mNCT, PS1-NTF, and Pen2 [SDS−PAGE (Figure 2c)] and by its proteolytic activity (Figure 2c, bottom).

Immunization of Mice with Purified γ-Secretase. Prior to immunization of mice, and to avoid any toxic effects of digitonin when it was injected in mice, we reconstituted the γ-secretase complex in phospholipid liposomes and removed digitonin as described in Materials and Methods and Figure 2d. The removal of detergent by gel filtration led to the formation of a precipitate, which was then pelleted by centrifugation. Similar amounts of γ-secretase were recovered in the supernatant and pellet as seen from the levels of components mNCT, PS1-NTF, and Flag-Pen2 (Figure 2d). Both fractions were thus used in parallel immunizations described in Materials and Methods, followed by monoclonal antibody selections.

Screening and Characterization of Monoclonal Antibodies Specific for γ-Secretase. Following immunization with γ-secretase, a total of 384 hybridoma clones were tested with an ELISA, dot blot, and live and fixed cell immunostaining (Figure S2 of the Supporting Information). Clone reactivity profiles were plotted for each screening method, and 12 clones were selected on the basis of their high reactivity to γ-secretase in all screens (Figure S2d of the Supporting Information, highlighted in green) and were subjected to further characterization (Figure S2e of the Supporting Information). First, Western blot analysis by SDS−PAGE revealed that clones 2D5 and 2G6 detected both mature and immature NCT bands in S20 total protein extracts, while clones 2F12 and 2H10 reacted strongly to subunit Aph1aL (Figure S2e of the Supporting Information, top). Next, BN-PAGE further revealed that all four clones reacted strongly to blots of native γ-secretase (Figure S2e of the Supporting Information, bottom). Clones 1C1, 1E3, 1C3, 1G9, 1E8, 2H8, and 2C7 showed marginal reactivity to denatured NCT in SDS−PAGE blots and mostly no interaction (except for 1C3) with γ-secretase in BN-PAGE blots (Figure S2e of the Supporting Information). The remaining clone, 2D7, did not produce significant signals in Western blots.

On the basis of their high reactivity in blots, antibody clones 2D5, 2G6, 2F12, and 2H10 were retained and their potential to immunoprecipitate native and denatured γ-secretase was examined. The antibodies were purified and coupled to Protein G agarose for IP experiments. As shown in Figure 3a, all effectively coprecipitated γ-secretase subunits NCT, PS1, Aph1, and Pen2 from native S20 extracts. Consistent with the SDS−PAGE analysis (Figure S2e of the Supporting Information), monoclonal antibodies 2D5 and 2G6 strongly coprecipitated NCT from denatured cell extracts containing dissociated γ-secretase, while 2F12 and 2H10 showed strong binding to Aph1 (Figure 3a). Under our experimental conditions, minor amounts of Aph1 were also precipitated by 2D5 and 2G6, most likely reflecting undissociated NCT−Aph1 subcomplexes.[34] No detectable amounts of PS1 or Pen2 were found in coprecipitates of denatured γ-secretase.

The antibodies were further characterized by cell imaging, and we found that our NCT-specific antibodies (2D5 and 2G6) strongly and unambiguously labeled γ-secretase in live S20 cells, in total contrast with the Aph1-specific antibodies (2F12 and 2H10), which stained only fixed and permeabilized cells (Figure 3b). Immunostaining by NCT- and Aph1-specific antibodies was mainly localized at the cell plasma membrane and in a single speckle within the protein trafficking and secretion system.

Epitope Mapping of γ-Secretase-Specific Antibodies. On the basis of the data described above, epitopes for antibodies 2D5 and 2G6 were localized in the γ-secretase subunit NCT, while 2F12 and 2H10 epitopes were mapped to Aph1. Interestingly, 2D5 and 2G6 target human epitopes, but not endogenous epitopes expressed by rodent cell lines (Figure 3c, top). In comparison, control antibody NCT164 was less specific, because it recognized all NCT orthologs. Using a set of NCT deletion constructs,[35] we narrowed the epitope localization for antibodies 2D5 and 2G6 to a 123-amino acid segment in NCT starting at position 41 (Figure 3d,f).

Surprisingly, antibodies 2F12 and 2H10 did not recognize endogenous Aph1 expressed in either human (HeLa and HEK)

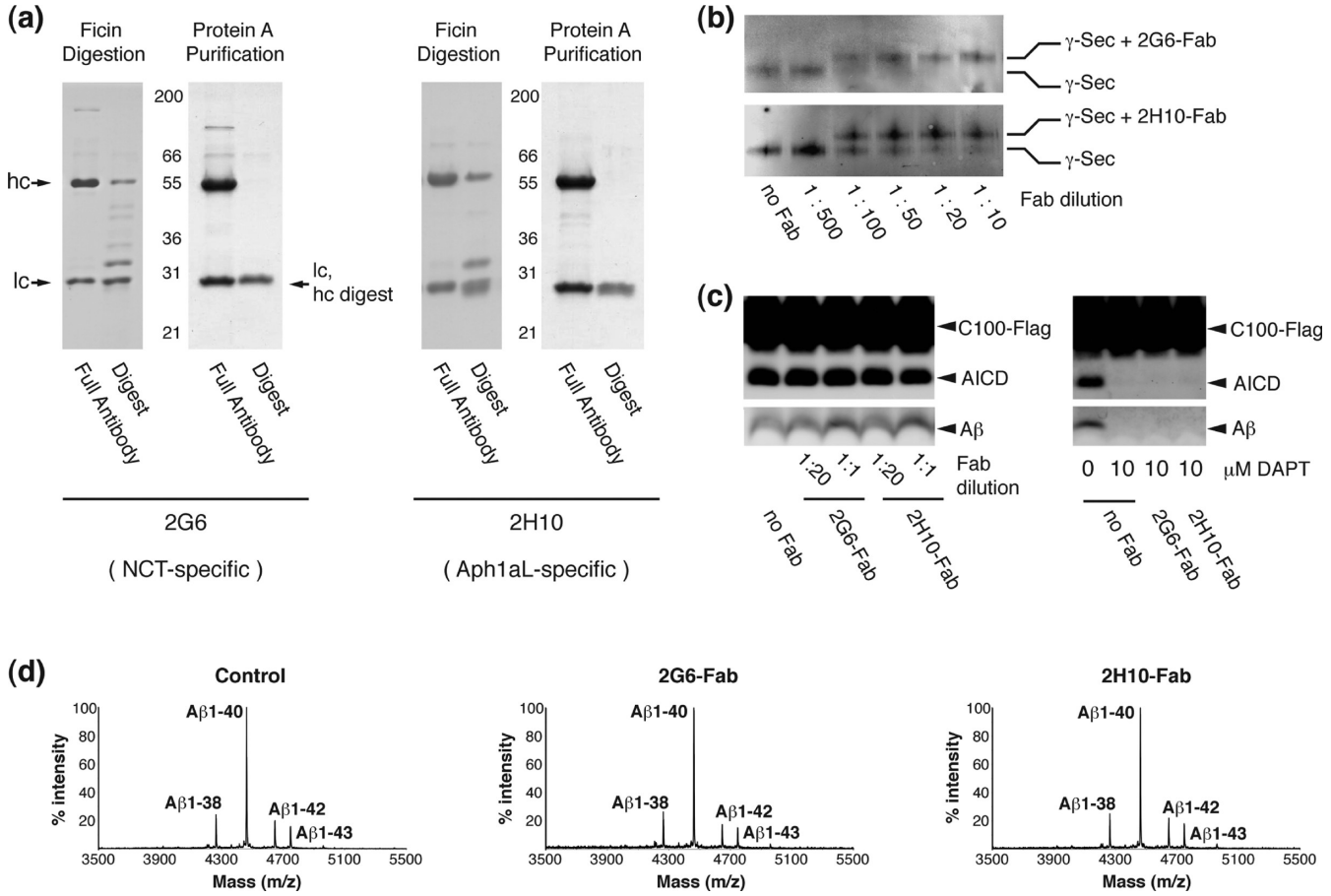

Figure 4. Production and characterization of Fab fragments specific for γ-secretase. (a) NCT- and Aph1-HA-specific monoclonal antibodies 2G6 and 2H10 were digested with immobilized ficin, and resulting Fab fragments were purified on a Protein A column to eliminate Fc fragments and undigested antibodies. Ficin digests and purified Fab fragments were analyzed by SDS−PAGE under reducing conditions followed by Coomassie staining. IgG light and heavy chains are indicated by lc and hc, respectively. (b) Dose-dependent binding of 2G6- or 2H10-Fab fragments was performed by incubating increasing amounts of the fragments with γ-secretase for 1 h. Complex formation was assessed by band shifts on BN-PAGE, in which γ-secretase was detected with Presenilin-specific antibodies MAB1563 and anti-PS1 in binding experiments with 2G6- and 2H10-Fab fragments, respectively. (c) The effect of Fab fragments on γ-secretase activity and its inhibition by DAPT was examined using cell-free assays. γ-Secretase was preincubated for 1 h with 2G6- or 2H10-Fab fragments prior to activity assays with (right) or without (left) γ-secretase inhibitor DAPT. (d) Aβ cleavage products analyzed by MALDI-TOF, with product peaks labeled according to human APP-C99 numbering.

or rodent (CHO and MEF) cell lines (Figure 3c, bottom), despite strong reactivity toward HA-tagged Aph1 (Figure S2e of the Supporting Information). Because S20 cells overexpress the Aph1aL ortholog of Aph1 fused to an HA tag, we tested whether the epitopes include all or part of the tag. Using a set of four, overlapped, synthetic peptides spanning the C-terminal end of Aph1aL-HA, we measured the binding of the antibody to the peptides by an ELISA (Figure 3e). Our monoclonal antibodies 2F12 and 2H10 recognized mainly the C-terminal 20-amino acid sequence of Aph1aL-HA and, to a lesser degree, the C-terminal 20-amino acid sequence of untagged Aph1aL (residues 246−265), but not the HA peptide alone. As expected, HA-specific antibody 12CA5 recognized equally the HA peptide and the C-terminal 20-amino acid sequence of Aph1aL-HA, which includes the HA sequence. In comparison, polyclonal antibody O2C2 strongly recognized the 20-amino acid sequence at the C-terminus of untagged Aph1aL (residues 246−265) and, to a lesser extent, the shorter Aph1aL peptides (residues 246−254). Interestingly, our data suggest that 2F12 and 2H10 target residues 255−265 of Aph1aL (Figure 3f), which are apparently not accessible under endogenous conditions, possibly because of post-translational modifications. Alternatively, the antibodies could be specific for a structural conformation adopted by residues 255−265 under the influence of the HA tag, which is not present in the endogenous protein.

Generation and Characterization of NCT- and Aph1-Specific Fab Fragments. Fab fragments specific for NCT (2G6-Fab) and Aph1-HA (2H10-Fab) were generated by ficin digestion of monoclonal antibodies 2G6 and 2H10, respectively, followed by purification on Protein A resin to eliminate Fc fragments and undigested antibodies (Figure 4a). Binding of Fab to native γ-secretase was confirmed by BN-PAGE, in which dose-dependent shifts of γ-secretase to higher molecular masses were observed following incubation with 2G6-Fab or 2H10-Fab (Figure 4b). Because formation of a complex between γ-secretase and specific Fab fragments may potentially affect γ-secretase properties, including its activity, we performed cell-free activity assays with γ-secretase preincubated with 2G6-Fab or 2H10-Fab. We found that the presence of these Fab fragments did not alter γ-secretase activity (Figure 4c, left) or its inhibition by potent compounds like DAPT (Figure 4c, right). The specificity of the Aβ cleavage profile was also not affected (Figure 4d).

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DISCUSSION

Relative to adherent cultures and taking into account time consumption, labor, and costs, our results clearly show that a substantial improvement in the production of purified γ-secretase is obtained by culturing S20 cells in suspension with ProCHO5 medium supplemented with only 1% FBS without the use of costly selection markers. Most significantly, our three-step approach to achieving a transition from plate to suspension cultures can be applied to other proteins expressed in mammalian systems, particularly membrane proteins and complexes.

As a result of our improved procedure, 10 L suspension cultures of CHO cells overexpressing functional γ-secretase are now routinely performed with a final yield of 0.8 mg of highly purified complex per culture. These amounts are sufficient to perform two-dimensional crystallization or conduct robot-assisted three-dimensional crystallization trials using nanoliter sample volumes, and potential in situ X-ray diffraction experiments.[36−38] The quantity and homogeneity of purified γ-secretase allow detection of a single band in Coomassie-stained Native gels (Figure 2a). Importantly, γ-secretase elutes as a single, well-resolved peak in gel filtration chromatography at an elution volume consistent with its Native-PAGE migration (Figure 2b), without an indication of higher-molecular mass aggregates. This highly purified γ-secretase retains its proteolytic activity and contains the mature γ-secretase components mNCT, Aph1, PS1-NTF, PS1-CTF, and Pen2 as seen in Coomassie-stained and immunoblotted SDS−PAGE (Figure 2b). We also found that the integrity and activity of γ-secretase are maintained when it is purified in CHAPSO, which is a detergent compatible with membrane protein crystallization.[15,32,33]

In addition to our mammalian expression and purification system, γ-secretase was reportedly expressed in Sf9 cells using baculovirus infection, and the complex was purified in amounts sufficient for single-particle analysis by electron microscopy.[39] However, it remains unclear whether the Sf9 approach can produce sufficient amounts to cover the needs of high-resolution methods.

Generation of monoclonal antibodies specific for γ-secretase is an important step for several applications, including production of Fab fragments as tools to aid the challenging process of crystallizing this membrane protein complex. γ-Secretase reconstituted in a phospholipid environment was well tolerated by mice during immunization, which produced several antibodies recognizing native γ-secretase as seen from immunoprecipitation experiments (Figure 3a), staining of live cells (Figure 3b), and BN-PAGE band shifts following formation of a complex between native γ-secretase and specific Fab fragments (Figure 4b). Four antibody clones were selected according to criteria of strong binding and high selectivity to γ-secretase components overexpressed in S20 cells. Two different epitopes were identified, of which one was unambiguously mapped to NCT segment 41−163 for antibodies 2D5 and 2G6. Our data revealed that the other epitope maps to amino acid residues 255−265 of Aph1aL, which is the Aph1 ortholog overexpressed in S20 cells. Interestingly, the corresponding antibodies, 2F12 and 2H10, did not detect endogenous Aph1aL in several cell types [HEK, HeLa, CHO, or MEF (Figure 3c)]. This observation suggests several explanations, including (1) endogenous inaccessibility of the epitope due to post-translational modifications, (2) endogenous removal of Aph1aL residues 255−265 by an unknown maturation process, and (3) conformational influence of the HA tag on the epitope.

Immunization with a protein is expected to generate antibodies against its most accessible and hydrophilic immunogenic regions.[40] Thus, our data strongly support a model in which the identified NCT and Aph1 epitopes are the most exposed regions of active γ-secretase embedded in a lipid bilayer, while PS1 and Pen2 are apparently less exposed or antigenic. Moreover, the topology of γ-secretase subunits NCT and Aph1 suggests that the epitopes identified in this study are located on opposite sides of the plasma membrane (Figure 3f[I]), which could explain the difference in live cell immunostaining between NCT- and Aph1-specific antibodies. Indeed, strong NCT-specific immunostaining is observed at the plasma membrane of live S20 cells (Figure 3b), which is compatible with a cell surface localization of γ-secretase and orientation of its NCT subunit toward the extracellular medium. In contrast, Aph1-specific antibodies do not stain live S20 cells but stain the cells after permeabilization and exposure of the cytoplasmic face of the plasma membrane to antibodies (Figure 3b).

In preparation for crystallization trials with γ-secretase, we have shown that our Fab fragments of NCT- and Aph1-specific antibodies are purified in a procedure that can be scaled up to amounts suitable for detailed structural studies. Interestingly, two synthetic antibodies in the Fab format were recently reported to target a different epitope in NCT.[41] Fab fragments provide extra protein−protein contacts, which are essential for protein crystallization. This is particularly relevant for membrane proteins because detergents in the protein preparation prevent a significant portion of the protein surface from forming contacts with adjacent molecules in the crystal lattice. Major breakthroughs have demonstrated the usefulness of Fab fragments in the crystallization of transmembrane systems, including G-protein-coupled receptors (GPCR) and ion channels.[14−17] Because it is important to determine whether artifacts are induced by Fab binding,[42] we assayed the proteolytic activity of γ-secretase preincubated with our NCT- or Aph1-specific Fab fragments and found no difference in comparison to its intrinsic activity (Figure 4c,d). In addition, the presence of Fab fragments did not prevent inhibition by DAPT, a potent γ-secretase inhibitor.[43] Therefore, three-dimensional γ-secretase structures determined with the help of 2G6-Fab or 2H10-Fab antibodies will likely reflect the active state of the enzyme complex.

In conclusion, we have adapted CHO cells overexpressing γ-secretase to growth in suspension, thus yielding multiliter cell cultures on a regular basis, and showed that a procedure for the purification of γ-secretase from those cells can be scaled up to produce milligram quantities of active γ-secretase. The amounts of protein were sufficient to reconstitute γ-secretase in liposomes for immunization of mice and production of monoclonal antibodies and purified Fab fragments specific for the complex. γ-Secretase nanocrystallogenesis can now be initiated, possibly in the presence of our specific Fab fragments that could be useful in facilitating crystal formation.

ASSOCIATED CONTENT

Supporting Information

Morphologies of γ-secretase-expressing CHO cells cultured in different media, screening of γ-secretase-specific antibodies, estimation of yields of purified γ-secretase, and a table of yield estimates of γ-secretase purified from a 10 L suspension culture.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.-R.A., C.S., M.C., M.D., M.O.-A., and P.C.F. designed the experiments. J.R.A., C.S., M.C., M.D., L.A., and M.O.A. performed the experiments. J.R.A. and P.C.F. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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