Generation of Alzheimer Disease-associated Amyloid $\beta_{42/43}$ Peptide by γ-Secretase Can Be Inhibited Directly by Modulation of Membrane Thickness[*][S]

Edith Winkler[‡§], Frits Kamp[‡§], Johannes Scheuring[¶], Amelie Ebke[‡§1], Akio Fukumori[‡§], and Harald Steiner[‡§2]

From the [‡]Adolf Butenandt Institute, Biochemistry, Ludwig Maximilians University Munich, Schillerstrasse 44, 80336 Munich, Germany, the [§]German Center for Neurodegenerative Diseases (DZNE), Schillerstrasse 44, 80336 Munich, Germany, and the [¶]Chemistry Department, Technical University Munich, Lichtenbergstrasse 4, 85747 Garching, Germany

Background: γ-Secretase-mediated intramembrane proteolysis generates A$\beta_{42/43}$, pathogenic peptides implicated in causing Alzheimer disease (AD).

Results: Reconstitution of γ-secretase in model membranes reveals that A$\beta_{42/43}$ generation can be lowered in thick membranes.

Conclusion: Membrane thickness is a crucial factor influencing the activity of γ-secretase to generate A$\beta_{42/43}$.

Significance: Targeting the lipid environment of γ-secretase by increasing membrane thickness may provide a therapeutic strategy for AD.

Pathogenic generation of amyloid β-peptide (Aβ) by sequential cleavage of β-amyloid precursor protein (APP) by β- and γ-secretases is widely believed to causally underlie Alzheimer disease (AD). β-Secretase initially cleaves APP thereby generating a membrane-bound APP C-terminal fragment, from which γ-secretase subsequently liberates 37–43-amino acid long Aβ species. Although the latter cleavages are intramembranous and although lipid alterations have been implicated in AD, little is known of how the γ-secretase-mediated release of the various Aβ species, in particular that of the pathogenic longer variants Aβ_{42} and Aβ_{43}, is affected by the lipid environment. Using a cell-free system, we have directly and systematically investigated the activity of γ-secretase reconstituted in defined model membranes of different thicknesses. We found that bilayer thickness is a critical parameter affecting both total activity as well as cleavage specificity of γ-secretase. Whereas the generation of the pathogenic A$\beta_{42/43}$ species was markedly attenuated in thick membranes, that of the major and rather benign Aβ_{40} species was enhanced. Moreover, the increased production of A$\beta_{42/43}$ by familial AD mutants of presenilin 1, the catalytic subunit of γ-secretase, could be substantially lowered in thick membranes. Our data demonstrate an effective modulation of γ-secretase activity by membrane thickness, which may provide an approach to lower the generation of the pathogenic A$\beta_{42/43}$ species.

Alzheimer disease (AD)[3] is the most common neurodegenerative disease affecting the elderly. Neurofibrillary tangles as well as amyloid plaques are invariant, major pathological hallmarks of the disease. The principal component of the amyloid plaques is the $\sim$4-kDa amyloid β-peptide (Aβ), which represents a heterogeneous mixture of hydrophobic peptides consisting of 37–43-amino acid Aβ species, Aβ_{40} being the major species (1). The longer variants Aβ_{42} and Aβ_{43} are highly aggregation-prone. Aβ_{42}, and as demonstrated recently Aβ_{43} as well (2), is highly pathogenic and believed to cause AD by triggering a pathogenic series of events, the so-called amyloid cascade that ultimately leads to neurodegeneration and dementia (1, 3). Aβ is generated by proteolytic processing of the β-amyloid precursor protein (APP), a type I membrane protein, by the combined action of β-secretase and γ-secretase (4). β-Secretase removes the large portion of the APP ectodomain and leaves a 99-amino acid C-terminal fragment in the membrane, which is subsequently cleaved by γ-secretase in the transmembrane domain (TMD). γ-Secretase cleavage occurs in a stepwise manner thereby liberating the APP intracellular domain (AICD) and the various Aβ species from the membrane (5, 6). The initial AICD-releasing cleavage at the ϵ-site occurs close to the cytoplasmic border of the membrane and is followed by further ζ- and γ-site cuts downstream the TMD ultimately releasing the different Aβ species, which become secreted (5, 6).

γ-Secretase belongs to the few unusual proteases that cleave their substrates within the core of the membrane (7). The protease is a complex composed of four integral membrane proteins, which are essential and necessary for activity (8–10). Pre-

[S] This article contains supplemental Materials and Methods, additional references, Figs. 1–4, and Table 1.
[1] Supported by the Elitenetzwerk Bayern.
[2] To whom correspondence should be addressed: Adolf Butenandt Institute, Biochemistry, Ludwig Maximilians University Munich, Schillerstrasse 44, 80336 Munich, Germany. Tel.: 49-89-2180-75480; Fax: 49-89-2180-75415; E-mail: harald.steiner@med.uni-muenchen.de.

[3] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-peptide; AICD, APP intracellular domain; APP, β-amyloid precursor protein; Bicine, N,N-bis(2-hydroxyethyl)glycine; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; FAD, familial AD; PC, phosphatidylcholine; PL, proteoliposome; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PS, presenilin; SUV, small unilamellar vesicle; TMD, transmembrane domain.

senilin (PS) 1 or its homolog PS2, respectively, act as catalytic subunits and mediate the intramembrane cleavages of the APP C-terminal fragment substrate (11). The potential substrate receptor nicastrin, APH-1 and PEN-2, are subunits required for stabilization and activation of PS (4, 12). Although drug targeting γ-secretase to lower Aβ generation may prove difficult as γ-secretase has many other substrates besides APP including Notch1, a major regulator of cell differentiation in development and adulthood (13), APP-selective inhibitors and modulators of γ-secretase have been developed that are in clinical trials for AD treatment (14).

Apart from the common sporadic forms, rare familial forms of AD (FAD) exist, which are characterized by an early disease onset and in the vast majority of the cases caused by mutations in PS1 and to a lesser extent by mutations in PS2. PS mutations cause an increase in the relative amounts of Aβ_{42} and/or Aβ_{43} species that are generated by γ-secretase cleavage (15, 16). Few FAD mutations have also been found in APP, most of them are located in the APP TMD at the γ-secretase cleavage sites and increase the ratio of Aβ_{42} to total Aβ (15).

Because APP processing is a membrane-associated process, alterations in the lipid membrane environment would naturally have an impact on the generation of Aβ and may thus potentially constitute a risk factor for AD. Alterations of a large variety of lipids have been documented in AD brain (17–19). However, whether and how known AD-associated lipid alterations affect APP processing and to what extent this may affect Aβ generation have remained unclear. Apart from previous studies demonstrating that amyloidogenic APP processing occurs predominantly in lipid rafts, *i.e.* cholesterol and sphingomyelin-rich membrane microdomains (20, 21), and is modulated by cholesterol (21–24), our knowledge on the lipid dependence of Aβ generation has remained limited (19, 25).

Reconstitution of purified membrane proteins has been an established and powerful tool to study directly the mechanism of action and structure-function relationships of a variety of integral membrane proteins including receptors, channels, carriers, as well as membrane-bound enzymes and enzyme complexes under well defined environmental conditions such as the membrane lipids in which the protein is embedded (26, 27). Recent advances in the purification of the enzymes involved in APP processing and their reconstitution in liposomes now provide a platform not only to identify lipids that modulate Aβ generation but also to study directly the mechanism by which lipids might affect Aβ generation. These studies already provided a first insight into the lipid classes, which affect β- and γ-secretase activities directly and identified, among other lipids, cholesterol as an activity-promoting lipid of both secretases (28, 29).

As has been reported for a number of membrane proteins and membrane protein complexes (30, 31), alteration of basic membrane properties such as hydrophobic thickness, chain order, and the packing of the lipids of the membrane core may likely modulate the activities of the secretases, in particular that of γ-secretase being a large intramembrane-cleaving protease complex containing 19 TMDs. To understand better the role of the lipid microenvironment of γ-secretase for the generation of Aβ, in particular that of the pathogenic A$\beta_{42/43}$ species, we set

out to investigate systematically whether and how acyl chain length as well as unsaturation of phosphatidylcholine (PC) lipids would influence γ-secretase activity and cleavage specificity in reconstituted proteoliposomes (PLs). Overall, we found that the activity of γ-secretase is directly affected by bilayer thickness, and we show that its modulation may be exploited to lower the generation of the pathogenic A$\beta_{42/43}$ species.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal and polyclonal antibodies, respectively, to Aβ_{1-16} (2D8) and to the N terminus of PEN-2 (1638) have been described (32, 33). Monoclonal and polyclonal antibodies to Aβ_{17-24} (4G8), to the ectodomain (clone 35), and to the C terminus of nicastrin (N1660) were obtained from Covance, BD Biosciences, and Sigma, respectively. Monoclonal anti-His$_5$ antibody was obtained from Qiagen. For additional reagents used, such as γ-secretase inhibitors (34), see supplemental Materials and Methods.

Cell Lines—Human embryonic kidney 293 (HEK293) cells, HEK293 cells stably expressing Swedish mutant APP (HEK293/sw) or coexpressing wt PS1, PS1 Δexon9, or PS1 L166P have been described before (35).

Lipids—Synthetic lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), diCn:0 PC, diCn:1(Δ9-cis) PC, Cn:0/Cm:0 PC, Cn:0/Cm:1 PC, diC18:2 PC, C18:0/C18:2 PC, C18:0/C20:4 PC, and C18:0/C22:6 PC) were purchased from Avanti Polar Lipids.

Preparation and Biophysical Characterization of Lipid Vesicles—12 mg of lipid was hydrated with 1.2 ml of distilled water for 1 h at room temperature. Hydrated lipids were sonicated with a Branson sonifier in pulsed mode (duty cycle 20%, output level 3) for 1 h at temperatures above the chain-melting temperature, T_m, of the lipids under a stream of argon gas. The clear suspension was centrifuged for 1 min with a table centrifuge to remove Ti particles and analyzed with dynamic light scattering (Malvern Instruments High Performance Particle Sizer) to confirm that small unilamellar vesicles (SUVs) with a diameter of about 30 nm had been formed. SUVs were diluted in assay buffer (35 mM sodium citrate, 3.5% glycerol, pH 6.4) to a final lipid concentration of 3.45 mg/ml and stored in aliquots of 200 μl at -20 °C until further use. For certain batches of diC14:0 PC, 15 mg of lipid was hydrated in 1.2 ml of distilled water to achieve robust activity of reconstituted γ-secretase.

Purification and Reconstitution of γ-Secretase into Lipid Vesicles—Native human γ-secretase was isolated from HEK293 cells by a multistep purification protocol as described before (36). The final enzyme preparations (Q-Sepharose eluates) contained 30–150 μg/ml protein in 5% glycerol, 1 mM EDTA, 1% CHAPSO, 25 mM caproic acid, 18.75 mM BisTris, 500 mM NaCl, pH 7.0. To reconstitute the enzyme into SUVs, 200 μl of the lipid vesicles were thawed, sonicated for 1 min as described above, and diluted 5 times with buffer (35 mM sodium citrate, 3.5% glycerol, 30 mM DTT, pH 6.4). Next, 1 volume of the purified enzyme was diluted with 4 volumes of the above prepared lipid vesicle suspension and incubated at 4 °C as described before (36) to allow the formation of PLs. The final PL mixture contained 6–30 μg/ml protein, 0.55 mg/ml lipids, and 0.2% CHAPSO (lipid/protein ratio (w/w): 20–100; detergent/lipid

ratio (w/w): 3.6, for all lipids used). In each experiment identical aliquots of the same enzyme preparation were taken. One aliquot was always used for the POPC control, and the other aliquots were used for the lipids under investigation.

γ-Secretase in Vitro Assay—Purified recombinant C100-His$_6$ APP substrate was prepared as described (9). In a standard assay, C100-His$_6$ was added to 20 μl of PLs to a final substrate concentration of 0.5 μM. Following overnight incubation at 37 °C, *in vitro* generated Aβ and AICD substrate cleavage products were analyzed by immunoblotting and quantified as described (36) by measuring the respective chemiluminescence signal intensities using the Fluorchem^{TM} 8900 detection system (Alpha Innotech).

Tris-Bicine Urea SDS-PAGE—To analyze individual Aβ species by immunoblotting, Tris-Bicine urea SDS-PAGE was used (37), which was adjusted for the separation of short Aβ species from substrate (36) by using a 11% separation gel.

For additional procedures, see supplemental Materials and Methods.

Statistical Analysis—For each lipid, independent enzyme reconstitutions using independent γ-secretase preparations and independent PC SUVs were analyzed. Quantitative data were collected from at least three independent assays. For some lipids, the corresponding data sets were repeatedly presented when used in more than one experimental context. Statistical significance of quantitative data were calculated by two-tailed unpaired Student's *t* test with Bonferroni correction for multiple testing.

RESULTS

To investigate the role of the lipid environment of γ-secretase on APP substrate processing we made advantage of our previously established cell-free reconstitution system comprised of purified enzyme, purified substrate, and lipid vesicles (36), allowing us to test the activity of the endogenous protease isolated from HEK293 cells in various defined model membranes. Because we had previously shown that our purified CHAPSO-solubilized γ-secretase displays robust activity and physiological cleavage specificity when reconstituted in PC vesicles (36), we based our studies on this lipid focusing on the influence of hydrophobic membrane thickness using model membranes of synthetic PC lipids with various acyl chains. POPC was used as control lipid in our studies because it represents the major phospholipid of biological membranes. Serving as general reference, a more detailed analysis of γ-secretase reconstituted in this lipid was undertaken, demonstrating robust enzyme activity, and as shown by electron microscopy and dynamic light scattering analysis, formation of PLs with a diameter of 30 nm (supplemental Fig. 1). We further found that consistent with earlier results (29), the reconstituted enzyme required the presence of residual detergent for activity, probably to allow the release of cleavage products from the PLs and/or to facilitate substrate availability (supplemental Fig. 2).

γ-Secretase Activity and Cleavage Specificity Depend on Membrane Thickness—The hydrophobic thickness of lipid membranes is determined by many parameters including temperature, the state of the lipids (liquid-crystalline or gel phase), the distance between the head groups, the length and the

amount of double bonds of the acyl chains, the angle of the acyl chains with respect of the plane of the bilayer, and the intercalation of the acyl chains (38). We started to investigate γ-secretase activity in PLs composed of PC with two monounsaturated acyl chains of increasing length allowing us to maintain the liquid-crystalline state of the bilayers at assay temperature. As in supplemental Fig. 1*A*, γ-secretase activity was generally analyzed after overnight incubation at 37 °C by immunoblot analysis of the formation of the cleavage products Aβ and AICD from purified recombinant APP C100-His$_6$ substrate added to the PLs. To assess changes in the whole profile of the individual Aβ species generated, Tris-Bicine urea SDS-PAGE was used, which allowed us to separate shorter (Aβ_{37-40}) and longer (A$\beta_{42/43}$) Aβ species from each other as well as from C100-His$_6$ (36).

As shown in Fig. 1*A*, only poor γ-secretase activity was observed with the short-chain lipids diC14:1 and di16:1, remaining below 5% of the POPC control. γ-Secretase activity increased sharply with diC18:1 PC, reached an optimum comparable with control with diC20:1 PC, and gradually decreased down to ~10% with the long chain lipids diC22:1 and diC24:1 PC.[4] Thus, γ-secretase activity depends on the length of the lipid acyl chains, with an optimum at 20 carbon atoms for monounsaturated acyl chains corresponding to a hydrophobic thickness of ~29 Å. We next investigated the cleavage specificity of γ-secretase with respect to the increase in the hydrophobic thicknesses of these bilayers. Assessment of the individual Aβ species generated revealed that the relative production of A$\beta_{42/43}$ was lowered with increasing acyl chain length (Fig. 1*B*). Remarkably, at bilayers thicker than di20:1 PC, *i.e.* thicker than a POPC bilayer, the A$\beta_{42/43}$/Aβ_{total} ratio dropped significantly below that found for the POPC control.

To substantiate further the modulatory influence of membrane thickness on the cleavage specificity of γ-secretase, we next analyzed saturated and monounsaturated PC in the C16–C18 acyl chain length range. As expected from our analysis above, substantial γ-secretase activity was observed for the diC16 lipids compared with the mixed-chain C16/C18 POPC control lipid, which was comparable with the diC18 lipid (Fig. 1*C*). In further support of the findings above, we found that compared with the diC16:0 membrane, the relative production of A$\beta_{42/43}$ by γ-secretase was increased in the thinner C16:0/C16:1 PC bilayer (Fig. 1*D*). Thickening the membrane by prolonging one of the acyl chains using C16:0/C18:1 PC (*i.e.* POPC) reverted this effect. Consistent with the data above, further thickening the bilayer using C18:0/C18:1 PC resulted in the lowest A$\beta_{42/43}$/Aβ_{total} ratio measured for these bilayers, lower than that produced by γ-secretase in POPC (Fig. 1*D*).

Taken together, we conclude that γ-secretase has an activity optimum for APP substrate cleavage in membranes with a hydrophobic core of 26–29 Å. The relative production of A$\beta_{42/43}$ species decreases with increasing thickness of the

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[4] To allow easy assessment of Aβ generation in relation to the thicknesses of the respective model membranes, the values of the hydrophobic thickness of the membranes were graphically represented for all data sets. These values were taken from the literature using well documented experimental data or were otherwise calculated (see supplemental Table 1).
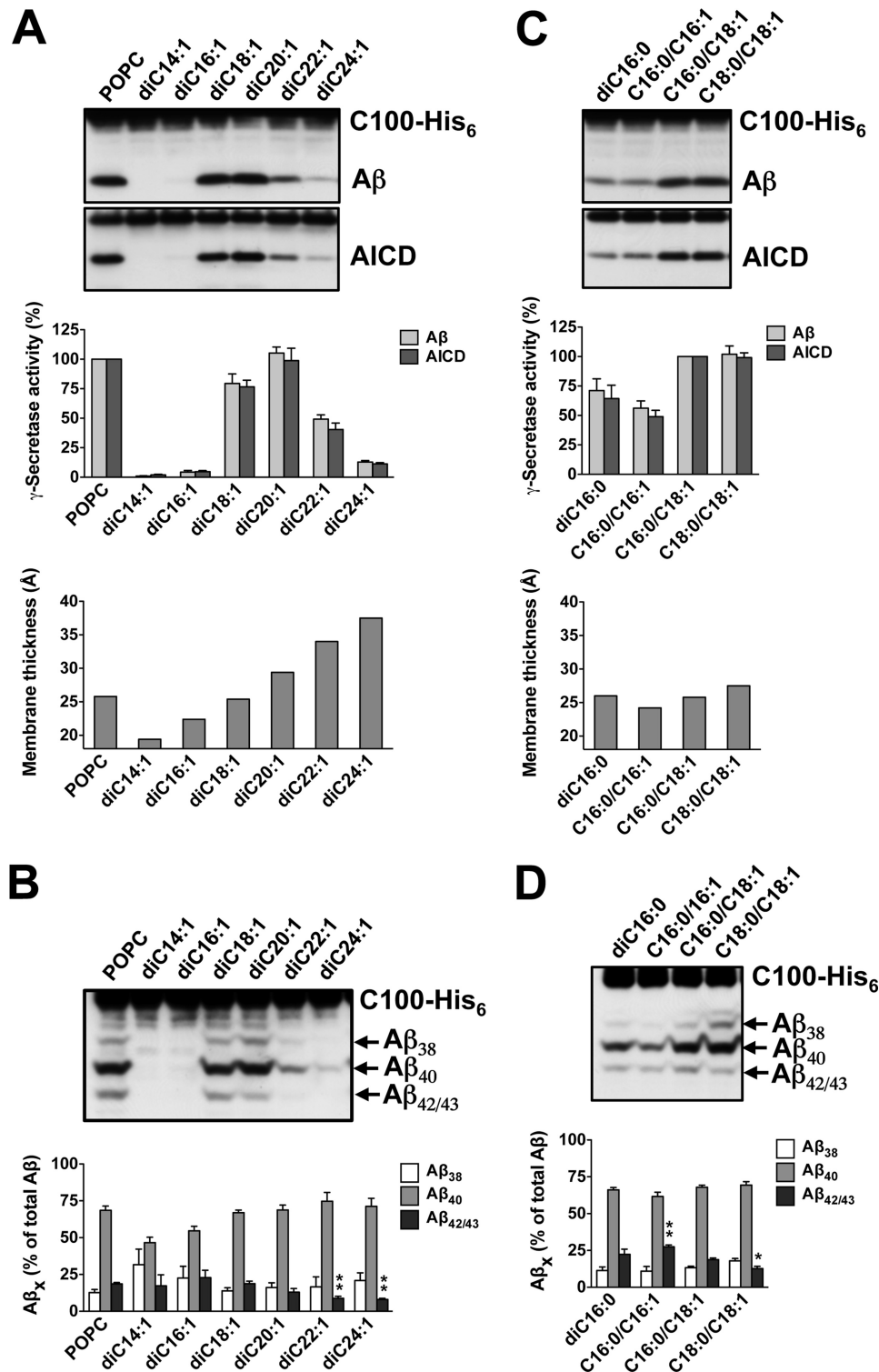
FIGURE 1. **Dependence of γ-secretase activity and cleavage specificity on membrane thickness.** *A*, γ-secretase activity was assessed following reconstitution of the purified enzyme into preformed SUVs composed of POPC or the indicated PCs containing monounsaturated acyl chains of increasing length. Upon addition of C100-His$_6$ substrate and overnight incubation at 37 °C, samples were analyzed for Aβ and AICD cleavage products by immunoblotting using antibodies 2D8 (Aβ) and anti-His$_5$ (AICD) (*top*). Quantitation of total Aβ and AICD cleavage products is shown in the *middle*. γ-Secretase activity was expressed relative to the control POPC PLs, which was set to 100%. Data are represented as mean ± S.E. (*error bars*) (POPC, $n = 7$; all other PCs $n = 3–7$). The hydrophobic membrane thickness of the bilayers comprising the corresponding lipids is shown in the *bottom panel*. *B*, aliquots of samples analyzed in *A* were subjected to Tris-Bicine urea SDS-PAGE to allow the separation of individual Aβ species and analyzed by immunoblotting using antibody 2D8 (*upper*). Note that Aβ$_{42}$ and Aβ$_{43}$ are not separated under our gel electrophoresis conditions and migrate as one band (Aβ$_{42/43}$). Quantitation of Aβ$_{38}$, Aβ$_{40}$, Aβ$_{42/43}$ species is shown in the *lower panel*. Data are represented as mean ± S.E. (POPC, $n = 7$; all other PCs, $n = 3–7$; **, $p < 0.01$ relative to POPC). *C* and *D*, conditions were as in *A* and *B* for diC16:0, C16:0/C16:1, C16:0/C18:1 (*i.e.* POPC), and C18:0/C18:1 PC. Quantitative data are represented as mean ± S.E. (POPC, $n = 13$, all other PCs, $n = 3–5$; *, $p < 0.05$; **, $p < 0.01$ relative to POPC).
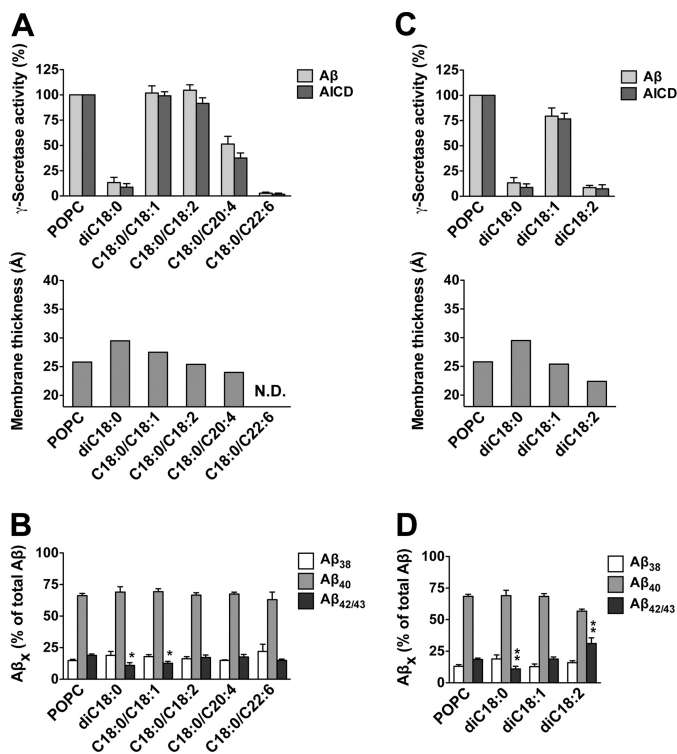
FIGURE 2. **Dependence of γ-secretase activity and cleavage specificity on lipid saturation state.** *A* and *B*, γ-secretase activity was assessed following reconstitution of the purified enzyme into preformed POPC, diC18:0, C18:0/C18:1, C18:0/C18:2, C18:0/C20:4, and C18:0/C22:6 PC SUVs as in Fig. 1, *A* and *B*. Quantitation of total Aβ and AICD and representation of hydrophobic membrane thicknesses are shown in *A* (*N.D.*, not determined), and quantitation of Aβ$_{38}$, Aβ$_{40}$, Aβ$_{42/43}$ species in is shown in *B*. Quantitative data are represented as mean ± S.E. (*error bars*) (POPC, *n* = 10, all other PCs, *n* = 3–6; *, *p* < 0.05 relative to POPC). *C* and *D*, conditions were as in *A* and *B* for POPC, diC18:0, diC18:1, and diC18:2 PC. Quantitative data are represented as mean ± S.E. (POPC, *n* = 11, all other PCs, *n* = 3–7; **, *p* < 0.01 relative to POPC).

FIGURE 3. **Increased Aβ$_{42/43}$ generation in diC14:0 membranes.** *A*, γ-secretase activity was assessed following reconstitution of the purified enzyme into preformed SUVs composed of POPC or the indicated PCs containing two saturated acyl chains of increasing length as in Fig. 1*A*. Quantitation of total Aβ and AICD as well as hydrophobic membrane thicknesses are shown in the *upper* and *lower panels*, respectively. Quantitative data are represented as mean ± S.E. (*error bars*) (POPC, *n* = 12, all other PCs, *n* = 3–7). *B*, aliquots of samples of *A* were analyzed as in Fig. 1*B*. Quantitative data are represented as mean ± S.E. (POPC, *n* = 12, all other PCs, *n* = 3–7; **, *p* < 0.01; ***, *p* < 0.001 relative to POPC). *C* and *D*, conditions were as in *A* and *B* for POPC, diC14:0, C14:0/C16:0, and C14:0/C18:0 PC. Quantitative data are represented as mean ± S.E. (POPC, *n* = 9, all other PCs, *n* = 3–7; **, *p* < 0.01; ***, *p* < 0.001 relative to POPC).

bilayer, suggesting that lipid chain length molulates not only total activity of γ-secretase, but also its cleavage specificity.

γ-Secretase Activity Is Affected by Lipid Saturation State— We next analyzed the impact of the saturation state of the lipid acyl chain on γ-secretase activity by comparing diC18:0, C18:0/C18:1, C18:0/C18:2, and two C18:0/polyunsaturated fatty acid PCs, *i.e.* lipids with an increasing degree of unsaturation in one of the acyl chains (Fig. 2*A*). γ-Secretase displayed poor activity in bilayers composed of diC18:0 PC, which might be due its gel state at the assay temperature (T_m of diC18:0 PC = 55 °C) (38). However, adding one or two double bounds in one of the acyl chains recovered γ-secretase activity toward the POPC control level. Arachidonic acid (C20:4 ω-6) reduced the activity to 50% of control, whereas docosahexaenoic acid (C22:6 ω-3) dropped the activity to <10%. As shown in Fig. 2*B*, while the Aβ$_{42/43}$ levels were not much different from POPC for most of these bilayers, the lowest productions of Aβ$_{42/43}$ were found for diC18:0 and C18:0/C18:1 PC, *i.e.* for the thickest bilayers of this series. Interestingly, addition of one double bond to both acyl chains (diC18:1 PC) also rescued the strongly reduced activity observed with diC18:0 PC, whereas addition of two double bonds in each acyl chain (diC18:2 PC) dropped the activity again to <10% (Fig. 2*C*). Consistent with the decrease in membrane thickness, Aβ$_{42/43}$ production was elevated with the latter lipid (Fig. 2*D*). Thus, overall,
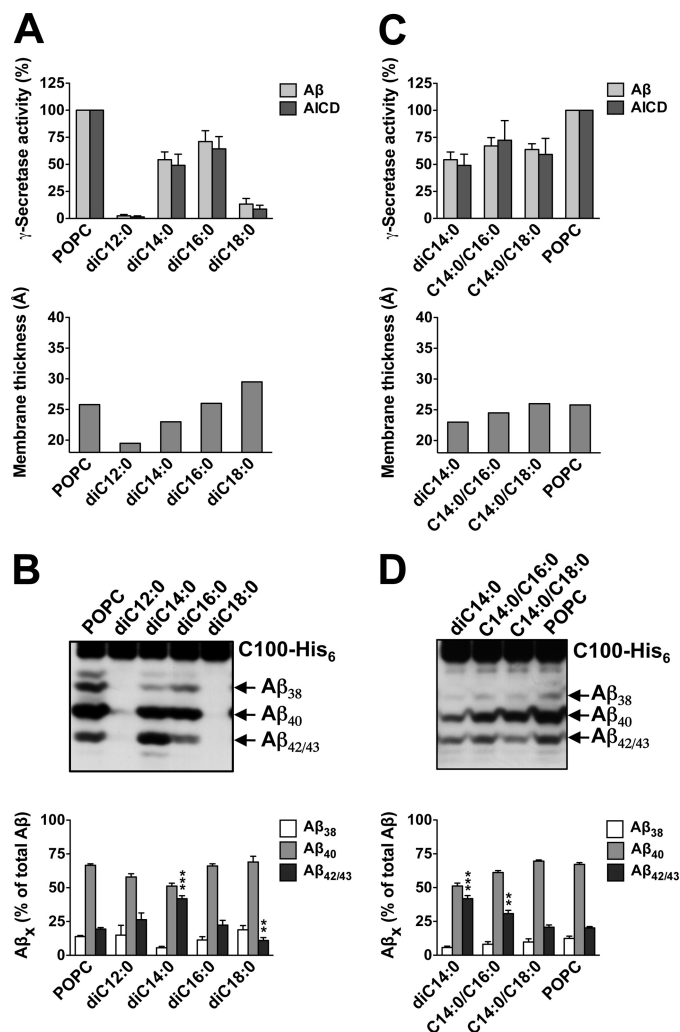
γ-secretase activity showed a trend of an increased relative production of Aβ$_{42/43}$ as the membrane thickness was reduced in consequence of an increasing degree of acyl chain unsaturation.

DiC14:0 PC Robustly Elevates Aβ$_{42/43}$ Generation, Which Can Be Reverted by Acyl Chain Length Elongation—We also studied the influence of bilayer thickening on γ-secretase activity in SUVs composed of PC containing two fully saturated acyl chains of increasing length. γ-Secretase activity was strongly reduced for diC12:0 PC, increased robustly for diC14:0 and diC16:0 PC, nearly reaching the activity of the POPC control, and as shown above, dropped strongly down again for diC18:0 PC (Fig. 3*A*). Whereas the profile of Aβ species produced by γ-secretase for diC16:0 PC was similar to that of the POPC

control, a striking change was observed for diC14:0 PC (Fig. 3*B*). In these thin bilayers, generation of the pathogenic A$\beta_{42/43}$ species was markedly increased, whereas Aβ_{38} and Aβ_{40} levels were reduced (Fig. 3*B*). As shown in supplemental Fig. 3, Aβ_{43} was the predominant pathogenic Aβ species generated by γ-secretase in this bilayer. In addition, the relative production of A$\beta_{42/43}$ was reduced as the membrane thickness increased from diC16:0 toward diC18:0 PC bilayers.

We next wondered whether the strikingly high relative production of A$\beta_{42/43}$ species found for γ-secretase reconstituted in diC14:0 PC could be reverted by elongating one of the acyl chains of this lipid thereby increasing the membrane thickness. As shown in Fig. 3*C*, γ-secretase displayed comparable activities in diC14:0, C14:0/C16:0, and C14:0/C18:0 PC bilayers. Indeed, the relative production of A$\beta_{42/43}$ was attenuated by C14:0/C16:0 PC and reached POPC control levels for C14:0/C18:0 PC (Fig. 3*D*). Thus, the increase in membrane thickness induced by the acyl chain elongation gradually normalized the "pathogenic" cleavage of γ-secretase in diC14:0 PC.

Increased A$\beta_{42/43}$ Generation by PS1 FAD Mutant γ-Secretases Is Attenuated in Thick Membranes—Having established that membrane thickness influences γ-secretase cleavage specificity modulating the generation of A$\beta_{42/43}$, we next investigated whether increasing membrane thickness could attenuate the pathogenic generation of A$\beta_{42/43}$ by FAD mutants within PS1, the catalytic subunit of γ-secretase. To this end, γ-secretase was purified from HEK293 cells stably expressing WT or FAD mutant PS1 and reconstituted in diC20:1-diC24:1 PC bilayers. FAD mutants of moderate (PS1 Δexon9) and strong (PS1 L166P) pathogenic activity (35) were chosen for analysis and changes in cleavage specificity were compared with WT PS1. Both FAD mutants showed the expected increases of A$\beta_{42/43}$ compared with the WT enzyme in POPC control conditions (Fig. 4). Comparable with their behavior in cultured cells, PS1 Δexon9 produced roughly similar amounts of A$\beta_{42/43}$ as Aβ_{40}, whereas the PS1 L166P mutant generated substantially more A$\beta_{42/43}$ than Aβ_{40}. In agreement with recent results (39), these FAD mutants generated higher amounts of Aβ_{43} than Aβ_{42} in the cell free assay system (supplemental Fig. 4). Increasing the lipid chain length caused a reduction of total activity for the PS1 Δexon9 mutant, which was accompanied by a change of the A$\beta_{42/43}$/Aβ_{total} ratios. In diC22:1 PC and diC24:1 PC lipids, A$\beta_{42/43}$ levels were lower than that of Aβ_{40}. Even for the strong PS1 L166P mutant, changes in the A$\beta_{42/43}$/Aβ_{total} ratios became apparent, which were most prominent in the diC24:1 PC bilayer. In this bilayer, a clear reduction of A$\beta_{42/43}$ was observed. Thus, the pathogenic activity of PS1 FAD mutants is attenuated in thick membranes and the increased production of A$\beta_{42/43}$ can be substantially attenuated under such conditions.

DISCUSSION

Despite being both an intramembrane-cleaving protease and a key AD drug target, only few studies have addressed the role of lipids for γ-secretase with respect to its activity so far (29, 40–42). Studies by Fraering *et al.* (40) and others (41, 42) showed that the purified detergent-solubilized protease is inactive unless it is reconstituted with membrane lipids. Investigating the influence of mixtures of PC with sphingolipids, or glyc-
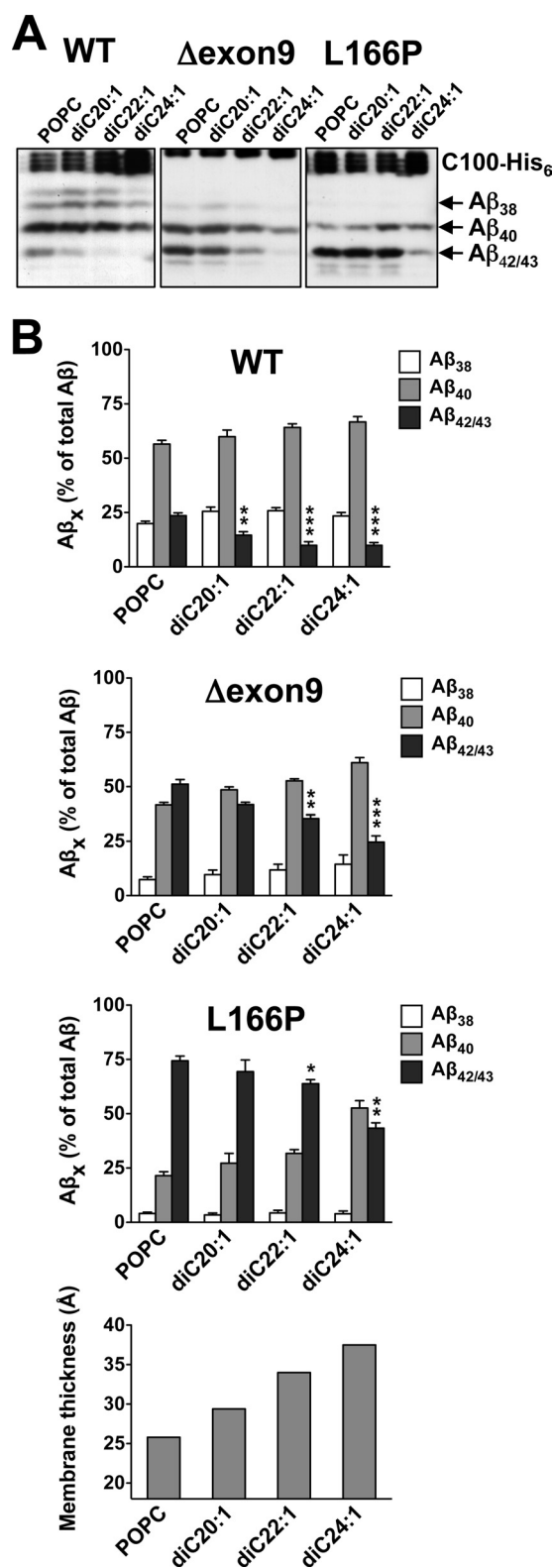

FIGURE 4. **Attenuation of the pathogenic activity of PS1 FAD mutants in thicker membranes.** *A*, γ-secretase cleavage specificity was assessed following reconstitution of the purified PS1 WT as well as Δexon9 and L166P FAD mutant enzymes into preformed POPC, diC20:1, diC22:1, and diC24:1 PC SUVs as in Fig. 1*B*. *B*, quantification of Aβ_{38}, Aβ_{40}, A$\beta_{42/43}$ species in samples of *A* and hydrophobic membrane thicknesses (*lower panel*) are shown. Quantitative data are represented as mean ± S.E. (*error bars*) (POPC, $n = 5$–8, all other PCs, $n = 3$–4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to the corresponding POPC).

erophospholipids containing different head groups, on the activity of γ-secretase, Osenkowski *et al.* (29) could further show that while the latter had little effect, sphingolipids could stimulate γ-secretase activity, which was boosted by soluble cholesterol.

Building on these initial studies, we systematically studied the role of membrane thickness and lipid chain order by reconstituting the purified enzyme into defined model membranes made of synthetic PC lipids with acyl chains of various length and saturation thereby allowing us to identify the optima for total activity and cleavage specificity of γ-secretase. γ-Secretase displayed robust (*i.e.* >75% compared with POPC) activity in membranes with a hydrophobic thickness of about 26–29 Å, almost regardless of whether PLs composed of C18:0/C18:1, C18:0/C18:2, diC18:1, or diC20:1 were used. The only exception was diC18:0 PC, a lipid in which γ-secretase displayed very low activity. It should be noted, however, that diC18:0 PC is the only lipid that we used which is in the rigid gel state at 37 °C. We thus conclude that a requirement for robust γ-secretase activity is that the lipid is in the liquid-crystalline state. Interestingly, lipids with polyunsaturated fatty acids, which are common in the brain, were not beneficial. Also adding double bonds in both acyl chains was not beneficial for its activity. Thus, besides hydrophobic thickness being an important parameter for robust γ-secretase activity, too many double bonds in the acyl chains of the hydrophobic core of the bilayer reduces γ-secretase activity. γ-Secretase displayed better activity when lipids were used with one saturated and one unsaturated acyl chain. POPC (C16:0/C18:1 PC) has one relatively stiff unsaturated and one more mobile saturated acyl chain (43) and apparently builds a bilayer with optimal hydrophobic thickness and packing of the lipids allowing a conformation of γ-secretase with optimal activity.

An important observation of this study was that the Aβ$_{42/43}$/Aβ$_{total}$ ratio profile observed for the various bilayers did not correlate with that of the corresponding total γ-secretase activity. Although there was an optimum membrane thickness for total Aβ production for POPC PLs, the tendency with regard to the Aβ$_{42/43}$/Aβ$_{total}$ ratio was that it decreased with increasing thickness of the bilayer. This was even observed for bilayers in which total activity of γ-secretase was very low. Interestingly, a decrease of Aβ$_{42/43}$ species was typically accompanied by an increase of the shorter Aβ$_{40}$ and/or Aβ$_{38}$ species, consistent with the sequential-cleavage model (5).

To distinguish the effects of membrane thickness from changes in lipid membrane fluidity on the cleavage specificity of γ-secretase, we used classes of phospholipids with similar membrane fluidity and lipid packing. Thus, we used PC with identical saturated acyl chains, saturated acyl chains with mismatching chain lengths, identical acyl chains having one *cis* double bond, and one saturated and one monounsaturated acyl chain. Within each class we found the same trend with regard to a reduced relative production of Aβ$_{42/43}$ for thicker membranes, justifying our conclusion that this effect is due to changes in thickness and not due to changes in fluidity. This is further illustrated in Fig. 5, which gives a summary of γ-secretase activity and cleavage specificity with respect to membrane
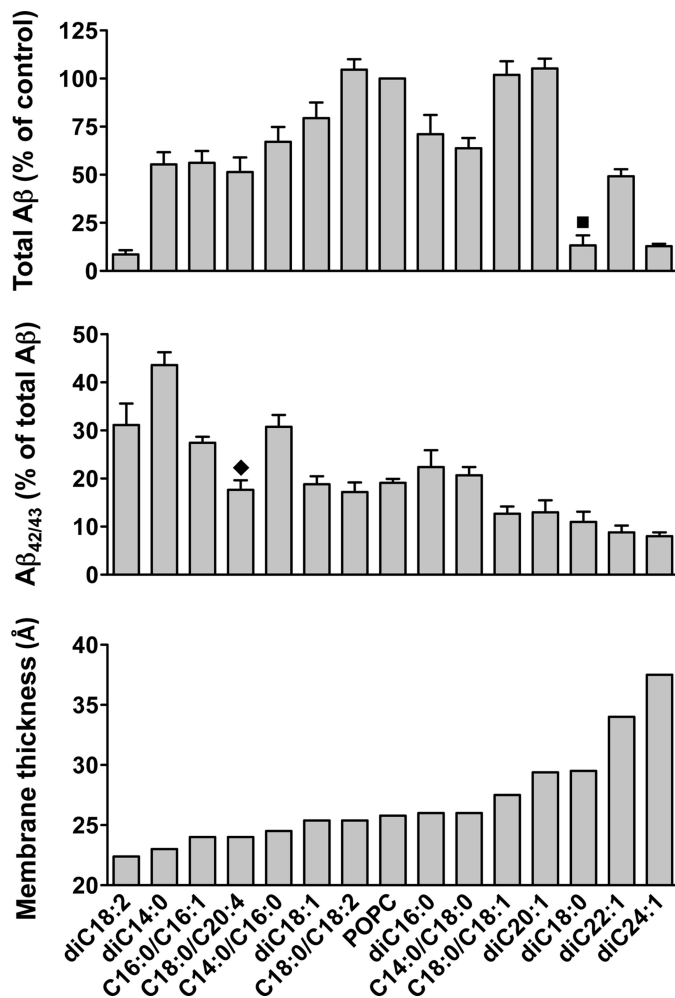

FIGURE 5. **Summary of the dependence of γ-secretase activity and cleavage specificity on membrane thickness.** Data of Figs. 1–3 were combined to represent all lipids in which the activity was higher than 5% compared with the POPC control. Total Aβ generation by γ-secretase in each lipid relative to the control POPC PLs is shown in the *top panel.* Activity is low for very thin and very thick membranes. Optimal activity is achieved for membranes with a thickness between 26 and 29 Å. Note that the activity varies for lipids with similar thickness probably due to differences in fluidity and lipid packing. Particularly lipids with one saturated and one unsaturated acyl chain have optimal activity. Relative Aβ$_{42/43}$ generation is shown in the *middle panel.* Aβ$_{42/43}$/Aβ$_{total}$ ratios decrease with increasing membrane thickness, irrespective to variations of total γ-secretase activity. The hydrophobic membrane thickness of the bilayers comprising the corresponding lipids is shown in the *bottom panel.* ■, diC18:0 PC is the only lipid investigated in our study, which is in the rigid gel state at 37 °C. ◆, estimation of the hydrophobic thickness of a bilayer composed of C18:0/C20:4 PC might be inaccurate due to the large mismatch of the C18:0 and C20:4 acyl chains (see supplemental Table 1).

thickness of in all used lipids in which the activity was >5% compared with the POPC control.

The highest Aβ$_{42/43}$/Aβ$_{total}$ ratio was found in diC14:0 PC bilayers. The Aβ$_{42/43}$/Aβ$_{total}$ ratio for diC14:0 PC PLs was about two times higher compared with the POPC control, similar to ratios found for weak FAD mutants. The high Aβ$_{42/43}$/Aβ$_{total}$ ratios found for diC14:0 PC PLs could be reverted to the values observed for the POPC control by increasing one of the acyl chains of the lipid. In these experiments, the total γ-secretase activity hardly changed whereas the Aβ$_{42/43}$/Aβ$_{total}$ ratios dramatically decreased. However, Aβ$_{42/43}$/Aβ$_{total}$ ratios were not as much elevated as in diC14:0 PC in other thin bilayers of

comparable thickness, such as diC18:2, or C16:0/C16:1 PC. This suggests that for diC14:0 PC, in addition to its property of forming a thin membrane bilayer, its two short saturated chains may lack the mobility to adjust to hydrophobic domains of the γ-secretase leading to a pathogenic change of the cleavage specificity of γ-secretase.

Because γ-secretase generated lower absolute and relative amounts of A$\beta_{42/43}$ when reconstituted in thicker membranes, we finally asked whether the same also applied for PS FAD mutant enzymes. As expected, when PS1 FAD mutant γ-secretase complexes were reconstituted in POPC vesicles, the A$\beta_{42/43}$/Aβ_{total} ratios were elevated compared with the WT PS1 γ-secretase control. Strikingly, A$\beta_{42/43}$/Aβ_{total} ratios were strongly attenuated when FAD mutants were reconstituted in thick membranes. Absolute A$\beta_{42/43}$ production was dramatically reduced for these PLs showing that the pathogenic activity of FAD mutant γ-secretase is strongly influenced by the thickness of its surrounding lipid bilayer and attenuated in thicker membranes. This was shown for both the PS1 Δexon9 and PS1 L166P FAD mutants, which display a moderate-strong elevation of A$\beta_{42/43}$ generation, respectively.

How does the lipid environment affect the activity of γ-secretase? Lipid properties affecting activities of integral membrane proteins include the head group, the chain order, the lipid packing, and the thickness of the hydrophobic part of the membrane. Particularly the extent of hydrophobic matching between the hydrophobic thickness of the bilayer and the length of the membrane-spanning part of the protein determines the amino acid composition, packing, and tilting of the TMDs of the protein thereby also influencing enzyme activity (30, 31). Interestingly, with respect to γ-secretase, changes of the tilt and elongation of the helical TMDs in bilayers of different thickness have recently been revealed by molecular dynamics simulations of the PS1 C-terminal fragment (44).

Altered hydrophobic matching between the bilayer and the TMDs of γ-secretase and/or of the substrate TMD and its surrounding membrane lipids might both influence the positioning of the substrate cleavage sites relative to the active site. As a result, an alteration of membrane thickness, which increases from early to late compartments of the secretory pathway (45–47), will directly affect hydrophobic matching and thereby not only influence total γ-secretase activity but also the ratios of Aβ_{40} *versus* the pathogenic Aβ_{42} and Aβ_{43} species generated. As shown in our study, the exposure of the γ$_{42/43}$ cleavage sites toward the catalytic site is apparently increased in thin membranes leading to an enhanced generation of A$\beta_{42/43}$, whereas in thicker membranes the γ$_{40}$ cleavage site is more exposed to the active site. Our data may thus also provide a direct explanation for the previously observed preferential generation of Aβ_{42} in the early compartments of the secretory pathway (48, 49).

Taken together, using defined model membranes, we have shown that γ-secretase activity and cleavage specificity toward its substrate APP are modulated by membrane thickness including inhibition of total Aβ generation and/or that of the pathogenic A$\beta_{42/43}$ species. Our findings thus suggest that alteration of membrane thickness to lower the generation of the pathogenic A$\beta_{42/43}$ species by γ-secretase could represent a target for potential therapeutic intervention of AD.

REFERENCES

1. Haass, C., and Selkoe, D. J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide. *Nat. Rev. Mol. Cell. Biol.* **8,** 101–112
2. Saito, T., Suemoto, T., Brouwers, N., Sleegers, K., Funamoto, S., Mihira, N., Matsuba, Y., Yamada, K., Nilsson, P., Takano, J., Nishimura, M., Iwata, N., Van Broeckhoven, C., Ihara, Y., and Saido, T. C. (2011) Potent amyloidogenicity and pathogenicity of Aβ43. *Nat. Neurosci.* **14,** 1023–1032
3. Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297,** 353–356
4. Lichtenthaler, S. F., Haass, C., and Steiner, H. (2011) Regulated intramembrane proteolysis: lessons from amyloid precursor protein processing. *J. Neurochem.* **117,** 779–796
5. Qi-Takahara, Y., Morishima-Kawashima, M., Tanimura, Y., Dolios, G., Hirotani, N., Horikoshi, Y., Kametani, F., Maeda, M., Saido, T. C., Wang, R., and Ihara, Y. (2005) Longer forms of amyloid β protein: implications for the mechanism of intramembrane cleavage by γ-secretase. *J. Neurosci.* **25,** 436–445
6. Takami, M., Nagashima, Y., Sano, Y., Ishihara, S., Morishima-Kawashima, M., Funamoto, S., and Ihara, Y. (2009) γ-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of β-carboxyl-terminal fragment. *J. Neurosci.* **29,** 13042–13052
7. Wolfe, M. S. (2009) Intramembrane-cleaving proteases. *J. Biol. Chem.* **284,** 13969–13973
8. Kimberly, W. T., LaVoie, M. J., Ostaszewski, B. L., Ye, W., Wolfe, M. S., and Selkoe, D. J. (2003) γ-Secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 6382–6387
9. Edbauer, D., Winkler, E., Regula, J. T., Pesold, B., Steiner, H., and Haass, C. (2003) Reconstitution of γ-secretase activity. *Nat. Cell Biol.* **5,** 486–488
10. Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003) The role of presenilin cofactors in the γ-secretase complex. *Nature* **422,** 438–441
11. Steiner, H. (2008) The catalytic core of γ-secretase: presenilin revisited. *Curr. Alzheimer Res.* **5,** 147–157
12. Dries, D. R., and Yu, G. (2008) Assembly, maturation, and trafficking of the γ-secretase complex in Alzheimer's disease. *Curr. Alzheimer Res.* **5,** 132–146
13. Wakabayashi, T., and De Strooper, B. (2008) Presenilins: members of the γ-secretase quartets, but part-time soloists too. *Physiology* **23,** 194–204
14. Imbimbo, B. P., and Giardina, G. A. (2011) γ-Secretase inhibitors and modulators for the treatment of Alzheimer's disease: disappointments and hopes. *Curr. Top. Med. Chem.* **11,** 1555–1570
15. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2,** 864–870
16. Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson-Wood, K., Lee, M., Seubert, P., Davis, A., Kholodenko, D., Motter, R., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George Hyslop, P., and Selkoe, D. J. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med.* **3,** 67–72
17. Han, X. (2005) Lipid alterations in the earliest clinically recognizable stage

of Alzheimer's disease: implication of the role of lipids in the pathogenesis of Alzheimer's disease. *Curr. Alzheimer Res.* **2,** 65–77

18. Hartmann, T., Kuchenbecker, J., and Grimm, M. O. (2007) Alzheimer's disease: the lipid connection. *J. Neurochem.* **103,** 159–170

19. Di Paolo, G., and Kim, T. W. (2011) Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat. Rev. Neurosci.* **12,** 284–296

20. Lee, S. J., Liyanage, U., Bickel, P. E., Xia, W., Lansbury, P. T., Jr., and Kosik, K. S. (1998) A detergent-insoluble membrane compartment contains Aβ *in vivo. Nat. Med.* **4,** 730–734

21. Ehehalt, R., Keller, P., Haass, C., Thiele, C., and Simons, K. (2003) Amyloidogenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts. *J. Cell Biol.* **160,** 113–123

22. Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998) Cholesterol depletion inhibits the generation of β-amyloid in hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6460–6464

23. Wahrle, S., Das, P., Nyborg, A. C., McLendon, C., Shoji, M., Kawarabayashi, T., Younkin, L. H., Younkin, S. G., and Golde, T. E. (2002) Cholesterol-dependent γ-secretase activity in buoyant cholesterol-rich membrane microdomains. *Neurobiol. Dis.* **9,** 11–23

24. Grimm, M. O., Grimm, H. S., Tomic, I., Beyreuther, K., Hartmann, T., and Bergmann, C. (2008) Independent inhibition of Alzheimer disease β- and γ-secretase cleavage by lowered cholesterol levels. *J. Biol. Chem.* **283,** 11302–11311

25. Zinser, E. G., Hartmann, T., and Grimm, M. O. (2007) Amyloid β-protein and lipid metabolism. *Biochim. Biophys. Acta* **1768,** 1991–2001

26. Rigaud, J. L., Pitard, B., and Levy, D. (1995) Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta* **1231,** 223–246

27. Seddon, A. M., Curnow, P., and Booth, P. J. (2004) Membrane proteins, lipids and detergents: not just a soap opera. *Biochim. Biophys. Acta* **1666,** 105–117

28. Kalvodova, L., Kahya, N., Schwille, P., Ehehalt, R., Verkade, P., Drechsel, D., and Simons, K. (2005) Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids *in vitro. J. Biol. Chem.* **280,** 36815–36823

29. Osenkowski, P., Ye, W., Wang, R., Wolfe, M. S., and Selkoe, D. J. (2008) Direct and potent regulation of γ-secretase by its lipid microenvironment. *J. Biol. Chem.* **283,** 22529–22540

30. Andersen, O. S., and Koeppe, R. E., 2nd (2007) Bilayer thickness and membrane protein function: an energetic perspective. *Annu. Rev. Biophys. Biomol. Struct.* **36,** 107–130

31. Lee, A. G. (2004) How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta* **1666,** 62–87

32. Shirotani, K., Tomioka, M., Kremmer, E., Haass, C., and Steiner, H. (2007) Pathological activity of familial Alzheimer's disease-associated mutant presenilin can be executed by six different γ-secretase complexes. *Neurobiol. Dis.* **27,** 102–107

33. Steiner, H., Winkler, E., Edbauer, D., Prokop, S., Basset, G., Yamasaki, A., Kostka, M., and Haass, C. (2002) PEN-2 is an integral component of the γ-secretase complex required for coordinated expression of presenilin and nicastrin. *J. Biol. Chem.* **277,** 39062–39065

34. Beher, D., Fricker, M., Nadin, A., Clarke, E. E., Wrigley, J. D., Li, Y. M., Culvenor, J. G., Masters, C. L., Harrison, T., and Shearman, M. S. (2003) *In vitro* characterization of the presenilin-dependent γ-secretase complex using a novel affinity ligand. *Biochemistry* **42,** 8133–8142

35. Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C., and Steiner, H. (2002) Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Aβ₄₂ production. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 8025–8030

36. Winkler, E., Hobson, S., Fukumori, A., Dümpelfeld, B., Luebbers, T., Baumann, K., Haass, C., Hopf, C., and Steiner, H. (2009) Purification, pharmacological modulation, and biochemical characterization of interactors of endogenous human γ-secretase. *Biochemistry* **48,** 1183–1197

37. Wiltfang, J., Smirnov, A., Schnierstein, B., Kelemen, G., Matthies, U., Klafki, H. W., Staufenbiel, M., Hüther, G., Rüther, E., and Kornhuber, J. (1997) Improved electrophoretic separation and immunoblotting of β-amyloid (Aβ) peptides 1–40, 1–42, and 1–43. *Electrophoresis* **18,** 527–532

38. Small, D. M. (1986) *The Physical Chemistry of Lipids: from Alkanes to Phospholipids* (Hanahan, D. J., ed), Plenum Press, New York

39. Quintero-Monzon, O., Martin, M. M., Fernandez, M. A., Cappello, C. A., Krzysiak, A. J., Osenkowski, P., and Wolfe, M. S. (2011) Dissociation between the processivity and total activity of γ-secretase: implications for the mechanism of Alzheimer's disease-causing presenilin mutations. *Biochemistry* **50,** 9023–9035

40. Fraering, P. C., Ye, W., Strub, J. M., Dolios, G., LaVoie, M. J., Ostaszewski, B. L., van Dorsselaer, A., Wang, R., Selkoe, D. J., and Wolfe, M. S. (2004) Purification and characterization of the human γ-secretase complex. *Biochemistry* **43,** 9774–9789

41. Zhou, H., Zhou, S., Walian, P. J., and Jap, B. K. (2010) Dependency of γ-secretase complex activity on the structural integrity of the bilayer. *Biochem. Biophys. Res. Commun.* **402,** 291–296

42. Wrigley, J. D., Schurov, I., Nunn, E. J., Martin, A. C., Clarke, E. E., Ellis, S., Bonnert, T. P., Shearman, M. S., and Beher, D. (2005) Functional overexpression of γ-secretase reveals protease-independent trafficking functions and a critical role of lipids for protease activity. *J. Biol. Chem.* **280,** 12523–12535

43. Huber, T., Rajamoorthi, K., Kurze, V. F., Beyer, K., and Brown, M. F. (2002) Structure of docosahexaenoic acid-containing phospholipid bilayers as studied by ²H NMR and molecular dynamics simulations. *J. Am. Chem. Soc.* **124,** 298–309

44. Sobhanifar, S., Schneider, B., Löhr, F., Gottstein, D., Ikeya, T., Mlynarczyk, K., Pulawski, W., Ghoshdastider, U., Kolinski, M., Filipek, S., Güntert, P., Bernhard, F., and Dötsch, V. (2010) Structural investigation of the C-terminal catalytic fragment of presenilin 1. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 9644–9649

45. Bretscher, M. S., and Munro, S. (1993) Cholesterol and the Golgi apparatus. *Science* **261,** 1280–1281

46. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell. Biol.* **9,** 112–124

47. Mitra, K., Ubarretxena-Belandia, I., Taguchi, T., Warren, G., and Engelman, D. M. (2004) Modulation of the bilayer thickness of exocytic pathway membranes by membrane proteins rather than cholesterol. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 4083–4088

48. Hartmann, T., Bieger, S. C., Brühl, B., Tienari, P. J., Ida, N., Allsop, D., Roberts, G. W., Masters, C. L., Dotti, C. G., Unsicker, K., and Beyreuther, K. (1997) Distinct sites of intracellular production for Alzheimer's disease Aβ₄₀/₄₂ amyloid peptides. *Nat. Med.* **3,** 1016–1020

49. Wild-Bode, C., Yamazaki, T., Capell, A., Leimer, U., Steiner, H., Ihara, Y., and Haass, C. (1997) Intracellular generation and accumulation of amyloid β-peptide terminating at amino acid 42. *J. Biol. Chem.* **272,** 16085–16088