Lipids as Modulators of Proteolytic Activity of BACE

IN VOLVEMENT OF CHOLESTEROL, GLYCOSPHINGOLIPIDS, AND ANIONIC PHOSPHOLIPIDS IN VITRO[8-10]

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The β-secretase, BACE, is a membrane spanning aspartic protease, which cleaves the amyloid precursor protein (APP) in the first step of proteolytic processing leading to the formation of the neurotoxic β-amyloid peptide (Aβ). Previous results have suggested that the regulation of β-secretase and BACE access to APP is lipid dependent, and involves lipid rafts. Using the baculovirus expression system, we have expressed recombinant human full-length BACE in insect cells and purified milligram amounts to homogeneity. We have studied partitioning of fluororoug-conjugated BACE between the liquid ordered and disordered phases in giant (10–150 μm) unilamellar vesicles, and found ~20% to associate with the raft-like, liquid-ordered phase; the fraction associated with liquid-ordered phase increased upon cross-linking of raft lipids. To examine involvement of individual lipid species in modulating BACE activity, we have reconstituted the purified BACE in large (~100 nm) unilamellar vesicles, and determined its specific activity in vesicles of various lipid compositions. We have identified 3 groups of lipids that stimulate proteolytic activity of BACE: 1) neutral glycosphingolipids (cerebrosides), 2) anionic glycerophospholipids, and 3) sterols (cholesterol).

Amyloid precursor protein (APP)2 is an abundant type I membrane protein with homology to glycosylated cell surface receptors (1) found in various mammalian tissues. Proteolytic processing of APP in human brain may give rise to the Aβ peptide, which is the major constituent of amyloid plaques in brains of patients suffering from Alzheimer disease (2, 3).

APP is a substrate for at least 3 proteolytic ("secretase") activities (4) designated α, β, and γ. The major proteolytic pathway, undertaken by ~95% of the APP in neurons, is α-γ, i.e. APP is first cleaved by a α-secretase within the Aβ region, and consequently by the γ-secretase. The second proteolytic pathway, which leads to the formation of Aβ, is the β-γ pathway. In this case, APP is first cleaved by the β-secretase (BACE, beta-site amyloid cleaving enzyme (5)) to allow further processing by the γ-secretase to produce the 4-kDa Aβ peptide.

Even though it is not the β-cleavage itself that would per se lead to the plaque formation, but rather the misfolding and aggregation of the generated peptide, up-regulation of the β-secretase activity is an issue in amyloidogenesis. Hence it is necessary to establish how access of the secretases to APP is regulated, and how are the proteolytic activities of the individual secretases modulated.

One obvious way of restricting a contact between two different membrane proteins is confining them into distinct cellular compartments, and/or possibly dispatching them to separate trafficking routes. To a certain extent, access of BACE and other secretases to APP may be limited in this way (both APP and BACE cycle between the cell surface and intracellular membrane compartments). Because trafficking largely relies on interacting proteins responsible for sorting, modifying (e.g. phoshorylation), and "packing" of the cargo, these are also candidates for involvement in β-cleavage regulation (6, 7). Another way to restrict contact between two different membrane proteins is by differential partitioning into distinct membrane microdomains, lipid rafts (8). Indeed, rafts had already been implicated in APP processing (9). Decreased levels of cholesterol and sphingolipids, both of which are indispensable constituents of lipid rafts, correlate with reduced β-cleavage (9), whereas exogenously added cholesterol seems to decrease α-cleavage (10); also ceramides, which have been suggested to play a role in subdomain organization and raft coalescence, were proposed as β-cleavage modulators (11). Besides, mutant BACE linked to a glycosylphosphatidylinositol anchor, which enhances its association with detergent-resistant membranes (DRMs), seems to cleave APP more efficiently than the wild type BACE (12).

There is a large body of experimental work done on cells in culture as well as ex vivo concerning partitioning of APP and BACE into DRMs (13–16). Despite the usefulness of probing DRM association in early stages of characterization of the proteins, and especially for detecting changes in interactions of membrane components, this type of experimental approach cannot provide adequate insight into raft connections to APP processing. Possibilities to manipulate lipid composition of the cell membranes are limited, and often do not deliver clear-cut results because the risk of secondary effects is high (disruption of the SNARE complexes required for exocytosis (17), block of the clathrin-coated pit formation (18, 19), phosphatidylinositol (4,5)-bisphosphate de localization from the plasma membrane (20)). In addition, β-secretase activity is likely to be regulated by direct modulation of the enzymatic activity of BACE. One such regulatory factor is the pH of the surrounding aqueous environment; another mechanism engages membrane lipids, either directly interacting with BACE as cofactors or costructures, or simply...
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providing optimal bulk membrane properties. In this paper we therefore set about to characterize the lipid requirements for 

BACE-linker-PreScission-TAP, which was cloned into the pFastBac (LEVLFQ sequence (SSGPSGS) followed by the PreScission protease cleavage site TAGCTCGAGCCCTTAAGCAGGGAGATGTCATCAGC). BACE1a was amplified by PCR with primers the introducing NcoI (con-peptide substrate for BACE (FS-1) was purchased from Bachem. Rabbit BACE ectodomain was from Oncogene, and the fluorogenic soluble other chemicals were from Sigma and Merck. The recombinant human beads, and the amino-reactive dye CY5 was purchased from Amersham Anatrace. OptiPrep density gradient media was from Axis-Shield (Oslo, Norway). Cell culture media and the NuPAGE 4–12% BisTris gels were from Invitrogen. IgG-Sepharose 6 Fast Flow, glutathione-Sepharose beads, and the amino-reactive dye CY5 was purchased from Amersham Biosciences, and the DiO fluorescent lipid analog as well as Alexa 488-cholera toxin subunit B conjugate were from Molecular Probes. Other chemicals were from Sigma and Merck. The recombinant human BACE ectodomain was from Oncogene, and the fluorogenic soluble peptide substrate for BACE (FS-1) was purchased from Bachem. Rabbit polyclonal antibody 7523 against BACE ectodomain was a kind gift from Christian Haass.

Constructs, Primers, and Baculovirus Generation—Human wile type BACE1a was amplified by PCR with primers introducing NcoI (containing initial ATG) site at the 5’ (CGTAGGCCATGGCCCAAGC- 

CCTGCCCTGGCTC) end and XhoI at the 3’ end (GGAATTCT- 

TAGCTCGAGCCCTTAAGCAGGGAGATGTCATCAGC). BACE was fused to a C-terminal TAP tag via a protease-resistant linker of the sequence (SSGPSGS) followed by the PreScission protease cleavage site (LEVLFQGP). The overall modular structure of the construct was thus BACE-linker-PreScission-TAP, which was cloned into the pFastBac vector (Invitrogen) under the control of the polyhedrin promoter. The recombinant baculovirus was generated according to the manufacturer’s instructions.

BACE Expression and Purification—All buffers used throughout the procedure were based on 50 mM HEPES, pH 7.25, 150 mM NaCl (HBS). The SF (Protein Sciences) cells were grown in suspension in a serum-free medium (SF900 II SFM) at 27 °C, and were infected with the recombinant baculovirus at the cell density of 1.5 × 10^6 cells/ml. The virus stock was roughly titrated by expression levels as judged from Western blotting. Cells were collected by centrifugation (30 min, 400 × g) 48 h post-infection, and frozen and stored at −80 °C.

BACE was purified from isolated membranes of the collected cells. Cells were homogenized in the presence of protease inhibitors (chymostatin, 6 mg/ml; leupeptin, 0.5 μg/ml; antipain 10 μg/ml; aprotinin, 2 μg/ml; pepstatin, 0.7 μg/ml; 4-aminophenylmethane sulfonyle fluoride; 10 μg/ml; and E64, 0.1 mM) in HBS buffer containing 0.25 M sucrose in a hand-held glass homogenizer (20–30 strokes). The homogenate was centrifuged at ~100,000 × g for 45 min at 4 °C. The supernatant was removed, and the resulting pellet was resuspended with the help of the glass homogenizer in HBS + 1% (w/v) dodecylmaltoside, supplemented with protease inhibitors. The lysate was incubated at room temperature with stirring for ~30 min, followed by centrifugation at ~100,000 × g for 45 min at 4 °C. The resulting supernatant was immediately loaded on an equilibrated IgG-Sepharose column (1.5-ml beads for 1-liter cell cultures) and washed with 10 column volumes of the running buffer (RB) (HBS + 5% glycerol, 0.5% (w/v) Triton X-100, pH 7.6), 1 column volume of RB + 5 mM ATP, 2 column volumes of RB, 2 column volumes of RB 2 mM EDTA, and finally 5 column volumes of RB. The flow was then stopped and ~150 μl of the PreScission protease (0.5 mg/ml) was added to the beads, mixed, and incubated either 3–4 h at room temperature or 12–16 h at 4 °C. The tagless protein was then eluted at a concentration of 0.25–0.5 mg/ml with RB supplemented with 10–50% glycerol. To remove the PreScission protease (glutathione S-transferase-tagged), the eluate was incubated with glutathione transferase beads for ~30 min, and the beads were removed by centrifugation. Purified BACE was stored at 4 °C, or mixed with 50% glycerol and stored at ~−20 °C, or frozen in liquid nitrogen for long-term storage.

Preparation of Large Unilamellar Vesicles (LUVs)—Large unilamellar vesicles were prepared from a hydrated suspension of multilamellar vesicles by extrusion. Briefly, lipids were mixed in chloroform in a borosilicate glass test tube, and the solvent was evaporated under a stream of nitrogen for ~1 h. The dry lipid film (which was consequently left under vacuum when the original volume of organic solvent was greater than 200 μl) was then hydrated with the LUV buffer (50 mM HEPES, 150 mM NaCl, 0.2 mM EDTA, pH 7.25), which was preheated to or above Tm of the lipid mixture. Hydration was carried out at or above the Tm with occasional vortexing for at least 30 min, or until the suspension appeared homogenous. The resulting suspension was subjected to 3–4 freeze-thaw cycles, and finally extruded through 100-nm pore diameter polycarbonate membrane using the Avanti mini-extruder.

Reconstitution of BACE to Form Proteoliposomes—Typically, LUVs were diluted to ~1 mg/ml, and the HEGA10 detergent was added to the final concentration corresponding to the "onset of solubilization" (23), which was determined by turbidity measurements. Typically, this value would be ~0.26% (w/v), differing slightly for each lipid mixture. The liposomes were then incubated for 10 min at room temperature to allow the detergent to equilibrate between the aqueous phase and the liposomes, and consequently, concentrated BACE (0.25–0.5 mg/ml in 0.4–0.5% Triton X-100 and 10–50% glycerol) was added at a protein to lipid ratio 1:70 to 1:150 (w/w). The Triton X-100 concentration was maintained safely below the critical micellar concentration of Triton X-100 (typically <0.008%). The mixture was then incubated at 32 °C with shaking for 15–45 min, and consequently for ~5 min at room temperature before loading on a Sephacry G-50 column of appropriate dimensions. The size of the column was determined empirically using liposomes with HEGA detergent, so that good separation of liposomes from HEGA detergent was ensured (as little as 0.01% HEGA can be detected using the BCA reagent). The proteoliposomes were collected (turbid fractions), adjusted to 12–20% iodixanol, and under-layered under the 2.5 and 5% iodixanol layers in an ultra-clear tube of an appropriate size depending on the final volume. Gradients in 11 × 34-mm tubes were centrifuged for 1 h and 15 min in a Beckman TLS55 rotor at 52 krpm at 4 °C; alternatively, 11 × 60-mm tubes were centrifuged in the Beckman SW60 rotor for 3 h at 42 krpm at 4 °C. Proteoliposomes would typically band at the 5/12% interface, or would be dispersed in the 5% layer. Empty liposomes would be mostly on the very top of the gradient.

The band was collected and diluted at least 6-fold with LUV buffer, and the proteoliposomes were pelleted for at least 2 h, 38 krpm, at 4 °C in the Beckman TLA45 rotor. The supernatant was carefully removed, and the pellets were resuspended in LUV buffer and stored on ice for several weeks without a detectable change in BACE activity.

Determination of BACE and Lipid Content in Proteoliposomes—An aliquot of the proteoliposomes was loaded on a NuPAGE BisTris 4–12% gel along with BACE mass standards, and run in MOPS buffer. The concentration was determined by densitometry after sil-
underwent fusion and formed large patches of membranes. Liposomes overnight. During membrane de-hydration at low temperature, the vesicles was deposited onto ITO-coated coverslips and put in the vacuum at 4 °C developed reconstitution technique was used (22). The vesicle suspension 

tures of high phase-transition temperature, membranes were first re-hy-

lipid concentration of 20 to 50 mM. To form the proteo-GUVs, a recently 

progression curve (typically up to 1500 s) measured as fluorescence at 

BACE in total brain lipid proteoliposomes.

enough to saturate 200 ng of BACE in 0.025% Triton X-100, or 40 ng of 

with a diameter varying between 10 and 150

GUVs preparation. After 3–4 h, numerous unilamellar vesicles formed, 


gm, 1 h) and resuspended in Milli-Q water containing 10 mM trehalose to 

lipozyme of 20 to 50 mM. To form the proteo-GUVs, a recently 

Electron Microscopy—Sample was dried on a grid, fixed with 2% paraformaldehyde, and quenched with glycine. For labeling, 7523 (C. Haass) antibody directed against the ectodomain was used together with 6-nm gold-conjugated secondary antibody. After labeling, the sample was fixed as before, washed, and stained with uranyl acetate.

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RESULTS

Expression and Purification of BACE—To prepare milligram amounts of active human BACE, we employed the baculovirus expression system along with the suspension-grown insect cell line Sf+ , which is capable of performing post-translational modifications common in mammalian cells (palmitoylation and glycosylation).

The polyhedrin promoter driven expression of BACE was first observed (as judged by Western blotting) at 40 h post-infection, and reached the highest levels at 48–60 h post-infection. More than 2 mg of homogenous (as judged by silver stained SDS-PAGE) preparation of tagless BACE, eluted at a concentration of 0.1–0.5 mg/ml, was obtained from 1 liter of 48-h post-infection culture (Fig. 1).

Recovery and Specific Activity of BACE Reconstituted in Total Brain Lipid Vesicles—The detergent-mediated reconstitution procedure presented here relies on incorporation of protein into preformed liposomes (23) and yields BACE reconstituted directionally (>80% ectodomain outside, supplemental Fig. S2) in large unilamellar vesicles; the yield was typically 15–30% of protein input by weight. The sidedness of the insertion did not depend on the lipid composition of the vesicles. Stable membrane-spanning insertion of BACE was confirmed by treatment with 0.1 μM NaN3, pH 11.3, followed by flotation in a density gradient. Virtually all BACE (as judged by silver stained SDS-PAGE) remained associated with the lipids (floating fraction, supplemental Fig. S3). In addition, we performed immunoelectron microscopy of the total brain lipid proteoliposomes. We observed vesicles ~50–200 nm in diameter, heavily labeled with anti-BACE antibody (Fig. 2). Virtually all immunodetected BACE was found to be membrane associated. The specific activity of BACE reconstituted in total brain lipid vesicles was at least 3-fold higher than that of the purified BACE in 0.02% Triton X-100, as

4 Specific activity of proteinase K in 0.5% Triton X-100 + 0.45% Tween 20 is comparable with detergent-free conditions (Qiagen product information).
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judged by cleavage of the soluble fluorogenic substrate, which mimics the cleavage site of the Swedish APP mutant.

Proteoliposomes consisting of BACE and total brain lipids were characterized by high reconstitution efficiency, reproducible activity, and stability (activity remains unchanged for several months when stored at 4 °C). BACE reconstituted in total brain lipid liposomes was therefore chosen as a standard, and reconstitution in total brain lipid liposomes was always performed as a control together with other samples. In this paper, we express specific activity of BACE as a percentage of the specific activity determined for brain lipid proteoliposomes.

Activity in Complex Lipid Mixtures—There are hundreds of lipid species present in cells, and the reasons for such diversity are poorly understood. One way to study this complexity is to analyze how lipids regulate activities of membrane proteins in a reconstituted system. We therefore dissected the total brain lipid extract, and attempted to elucidate which lipid species are responsible for supporting BACE activity.

As a control, a mixture mimicking the total brain lipid extract was mixed from individual components based on the known glycerophospholipid composition (data from Avanti catalog) and estimated amounts (TLC) of SM, glycosphingolipids, and cholesterol. The composition was as follows: PC:PE:SM:cerebrosides:cholesterol:gangliosides:PS:PA:PI (10:17:27:10:15:10:15:3:2, w/w). BACE activity in this mixture was determined to be 95 ± 12% of TBLE (total brain lipid extract).

Because interpretation of the effects caused by a particular lipid species in complex lipid mixtures such as total brain lipids is problematic, we proceeded to investigate BACE activity in various simpler lipid mixtures of defined head group compositions, ranging from simple pseudoternary mixtures to more complicated mixtures of up to 6 components.

Specific Activity of BACE Reconstituted in Glycero- and Glycerophospholipid:Cholesterol Vesicles—Glycerophospholipids are the most abundant phospholipids in living cells, of which phosphatidylcholine accounts typically for up to 50% in mammalian cells. Specific activity of BACE in pure PC (brain PC or synthetic POPC) vesicles was not determined because of extremely low recoveries (<5%). It was clear, however, that specific activity in PC vesicles was severalfold (at least 3–5-fold) lower than that of BACE in TBLE vesicles.

Recoveries improved somewhat when cholesterol was included, therefore POPC:cholesterol (2:1, mol/mol), was used instead. Specific activity of BACE in POPC:cholesterol vesicles is ~5-fold lower than in TBLE vesicles (Fig. 3). It has been shown that membrane proteins often require PE for activity, PE serving as a “chaperone” (28) and, along with other “nonbilayer lipids,” PE seems to be important for maintaining the physical state of the bilayer, allowing the membrane to readily undergo local rearrangements in response to changes in external conditions (27).

However, in the case of BACE, including 20% PE did not lead to any significant increase in BACE activity (26 ± 12% relative to TBLE, Fig. 3).

On the other hand, there was a strong effect of negatively charged phospholipids on BACE activity. PC:PS (80:20, w/w) supported BACE activity to 58 ± 4% of TBLE, whereas mixtures of PC:PE:PS and PC:PE:PA (60:20:20, w/w) brought the specific activity of BACE virtually to the level of TBLE, resulting in 99 ± 9 and 135 ± 31% of TBLE activity, respectively (Fig. 3).

Specific Activity of BACE Reconstituted in Vesicles Composed of Glycero- and Glycerophospholipids, (Glyco)sphingolipids, and Cholesterol—Sphingolipids and cholesterol are essential components of lipid rafts, and various pseudo-tetra-mixtures of PC, SM, and cholesterol mixed at ratios that allow for ld-lo phase coexistence, have been shown to be useful to imitate lipid rafts. Specific activity of BACE in the ld phase forming mixture of PC:SM:cholesterol (1:1:1 or 2:2:1 mol/mol) did not dramatically differ from POPC:cholesterol, resulting in 29 ± 16% of TBLE activity.

To assess the role of cholesterol in modulating membrane properties relevant to BACE activity, we used proteoliposomes consisting of PC:PE:SM:gangliosides supplied with increasing amounts of cholesterol (0, 7.5, and 15%, w/w) substituting for SM (25, 17.5, and 10% w/w) final to yield cholesterol:sphingolipid molar ratios of 0.4:2.5. There was an increase in BACE activity along with increasing cholesterol:sphingolipid molar ratios as follows: 18 ± 1% for PC:PE:SM:gangliosides (50:20:

When sphingolipids in this mixture (SM and gangliosides) were replaced with cerebrosides (PC:PE:cerebrosides:cholesterol, 50:20:15:15 (w/w), mixture 1 in TABLE ONE), specific activity was determined to be 46 ± 4%. Even though this increase is relatively small, the trend seems to be confirmed in another mixture containing both cerebrosides (12%) and a small amount of SM (5%), which is present at the expense of lowering PC content accordingly (PC:PE:SM:cerebrosides:cholesterol, 45:20:5:15:15 (w/w), mixture 3), in which BACE displays specific activity of 58 ± 6%, representing ~20% increase (Fig. 4B). In addition, we found that cerebrosides, as well as synthetic C8-glycosylceramide, greatly enhance the activity of purified soluble ectodomain of BACE in the presence of Triton X-100 (~20 nM BACE in the presence of 0.1 mM cerebrosides or C8-glycosylceramide displays almost 9- or 4-fold higher activity than a control in Triton X-100 only. Such an increase was only observed with cerebrosides and glucosylceramide, and not with any other lipids in the solubilized state. However, only a mild effect was observed with the full-length BACE (~1.2-fold increase with 0.1 mM
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cholesterol:PS. Comparing {0,2,4,10} with {3,5,6,8} revealed higher activities in the mixtures that included cerebrosides.

With PA, the situation is somewhat different. Introducing 5% PA to a mixture of PC:PE:SM:cerebrosides:cholesterol promoted activity more notably than 8% PS (compare {7,3,6}). However, when in this case cerebrosides are omitted, the resulting mixture [9] displays high activity.

Activity in Cholesterol-depleted Proteoliposomes—Methyl-β-cyclo-dextrin is often used for cholesterol depletion from cell membranes. Using this same approach on proteoliposomes allows us to distinguish between the requirement for cholesterol to maintain the activity of reconstituted BACE as opposed to the requirement for cholesterol presence during membrane insertion of BACE. When cholesterol was depleted either from total brain lipid proteoliposomes, or from liposomes before BACE insertion by means of methyl-β-cyclo-dextrin treatment (~5 min), activity of BACE was dramatically reduced (~4-fold) in comparison with untreated control, whereas there was no effect on non-reconstituted solubilized BACE (Fig. 6).

Partitioning Properties of BACE in Giant Unilamellar Vesicles—BACE has been previously found to be partially DRM-associated (15), however, DRM association does not directly imply raft association. We therefore chose to approach the issue of putative raft association of BACE by probing the partitioning of BACE between the lo and ld phases in GUVs.

As expected, distribution of BACE in pure DOPC vesicles (ld phase only) is homogenous (Fig. 7A). However, we found that in a system with coexisting ld-lo phases BACE does indeed partition into the lo phase. In GUVs composed of DOPC:brain SM:cholesterol (2:2:1, mol/mol) (this mixture gives rise to large, microscopic domains of several mm in diameter), about 15–20% of BACE partitions into the lo phase, as judged from the fluorescence correlation spectroscopy measurements and confocal imaging (Fig. 7B). When 1 mol % of GM1 (or more) is included in the membrane, then upon cross-linking with cholera toxin subunit B, redistribution of BACE occurred, BACE being almost equally distributed between ld and lo phases (Fig. 7, E and F). This may reflect a specific interaction of BACE with GM1. Presence of GM1 or brain gangliosides at ~2% mol/mol did not lead to any observable change in the specific activity of BACE in large proteoliposomes. However, because we are aware of the putative role of GM1 in amyloidogenesis (see for example, Refs. 24 and 25), the engagement of GM1 or other gangliosides directly in regulating β-cleavage will be further addressed in future experiments focusing on the interaction of BACE with full-length APP rather than with the peptide substrate.

DISCUSSION

Being an integral membrane protein, the aspartic protease BACE is likely to respond to its lipid environment on a molecular level (conformation, oligomerization, intramolecular charge distribution etc.) in a way that could eventually affect its activity. In this study we investigated the dependence of the proteolytic activity of BACE (irrespective of APP as the substrate, but using a soluble peptide substrate), on the composition of the lipid bilayer in a reconstituted system consisting of purified full-length BACE and defined lipids.

Even though the soluble BACE ectodomain alone displays proteolytic activity, a higher specific activity of the full-length BACE has been reported (26). Westmeyer et al. (26) proposed the higher specific activity of the full-length protein to be due to dimerization, which would occur exclusively when the ectodomain is attached to the membrane. In contrast to these authors, using blue native-PAGE, we detected dimers of the purified recombinant ectodomain of BACE as well as oligomers of the full-length BACE (supplemental Fig. S4). Dimerization thus does not seem to be strictly dependent on membrane attachment, which

glucosylceramide over the control). This may be because of the interference of Triton X-100, because higher concentrations are necessary for full-length BACE. The effect of cerebrosides was further investigated in mixtures including anionic phospholipids.

Specific Activity of BACE Reconstituted in Vesicles Composed of Glycero-phospholipids, (Glyco)sphingolipids, Cholesterol, Including Negatively Charged Glycerophospholipids—Mixtures listed in TABLE ONE will be referred to by their numbers enclosed in braces (|) in this paragraph.

In pure glycero-phospholipid vesicles, anionic phospholipids (PS and PA) promoted BACE activity notably. In vesicles composed of glycero-phospholipids, sphingolipids, and cholesterol, the outcome was dependent on the sphingolipid content of the mixture. In otherwise identical mixtures (either with {3,6,8} or without {0,2,4,10} cerebrosides), there was a clear trend of increasing BACE activity with increasing amounts of PS (Fig. 5). However, in mixtures with different sphingolipid content, the response to the addition of PS varied (compare {2,4,5,6}). Thus the ratio of sphingolipid to phospholipid (or perhaps cholesterol) content may play a role as well. We further asked if cerebrosides contribute to supporting activity in mixtures of PC:PE±SM:
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TABLE ONE

Results and compositions for lipid mixtures consisting of glycerophospholipids, sphingolipids, and cholesterol

Composition is given in mol %. All lipids were of brain origin except for cholesterol (sheep wool) and PA (produced from egg PC). BACE was reconstituted in LUVs of given compositions by detergent-mediated reconstitution followed by flotation in a density gradient to separate resulting proteoliposomes from unconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture was measured. Activity represents specific activity of BACE in % relative to BACE reconstituted in total brain lipid proteoliposomes. The value represents the average of results obtained from 2 to 7 independent reconstitution experiments.

| Mixture | PC | PE | SM | Cerebroside | Cholesterol | PS | PA | Activity | Mean ± S.E. |
|---------|----|----|----|-------------|-------------|----|----|----------|-------------|
| 1       | 44 | 18 | 0  | 12          | 26          | 0  | 0  | 46       | 4           |
| 2       | 35 | 18 | 14 | 0           | 26          | 8  | 0  | 51       | 3           |
| 3       | 40 | 18 | 5  | 12          | 26          | 0  | 0  | 58       | 6           |
| 4       | 26 | 18 | 23 | 0           | 26          | 8  | 0  | 61       | 5           |
| 5       | 35 | 18 | 5  | 12          | 26          | 8  | 0  | 62       | 2           |
| 6       | 31 | 18 | 5  | 12          | 26          | 8  | 0  | 67       | 5           |
| 7       | 35 | 18 | 5  | 12          | 26          | 8  | 5  | 78       | 4           |
| 8       | 22 | 18 | 5  | 12          | 26          | 17 | 0  | 97       | 7           |
| 9       | 46 | 18 | 5  | 0           | 26          | 0  | 5  | 98       | 12          |
| 10      | 34 | 18 | 5  | 0           | 26          | 17 | 0  | 64       | 10          |
| “0”    | 44 | 18 | 9  | 0           | 26          | 2  | 0  | 36       | 1           |

FIGURE 5. Specific activity of BACE in vesicles composed of PC, PE, cholesterol, SM, ±cerebrosides, and ±PS/±PA. Anionic phospholipids and cerebrosides affect BACE activity. Activity increases with increasing PS content in proteoliposomes of otherwise identical compositions, and cerebrosides included at the expense of PC in proteoliposomes of otherwise identical compositions further stimulate the activity. BACE was reconstituted in LUVs by detergent-mediated reconstitution followed by flotation in a density gradient to separate the resulting proteoliposomes from unreconstituted protein. BACE content was determined in each sample, and specific activity of BACE in each particular mixture was measured and is expressed in % relative to the specific activity of BACE in total brain lipid proteoliposomes (=100%). In all mixtures, PE is present at a concentration of 18% mol/mol (20% w/w), cholesterol at 26% mol/mol (15% w/w), and SM at 5% mol/mol, except for samples “0” (9% SM) and “2” (14 mol % SM). Where applicable, cerebrosides are present at a concentration of 12 mol%. Amounts of PS/PA are indicated. The numbers on the bars indicate the mixture number in TABLE ONE.

FIGURE 6. Specific activity of BACE in proteoliposomes after treatment with methyl-β-cyclodextrin. Proteoliposomes with reconstituted BACE and control (BACE in Triton X-100) were treated with 5 mM methyl-β-cyclodextrin (mbCD) for 15 min. Activity was then measured after dilution with the assay buffer. Activity in non-treated controls was set to 100%.

We hypothesized that lipids differ in their capacities to support enzymatic activity of BACE. Our data, 1) back up the concept of a multiple lipid system being generally more capable of supporting protein activity; and 2) suggest lipid candidates involved in affecting BACE activity. We focused on investigating effects of lipids defined by their head groups, and did not examine impact of acyl chain composition.

All cell membranes comprise anionic glycerophospholipids: PA, PI, and PS, the latter two being confined almost exclusively to the cytoplasmic leaflet of the membrane. Negatively charged lipids have been identified as regulators of function of numerous integral membrane proteins (glucose transporter, Ca$^{2+}$-ATPase, (Na$^+$,K$^+$)-ATPase (Ref. 29 and references therein, Ref. 30), nicotinic acetylcholinesterase receptor (Ref. 29 and references therein)).

We demonstrated a strong effect of PS and PA on BACE activity, which is further enhanced by PE. We can envisage four possible, mutually not exclusive, ways, in which PS or PA could stimulate proteolysis by BACE: electrostatic interactions at the membrane interface, a direct interaction of BACE with a negatively charged lipid in the outer leaflet, a direct interaction of the cytoplasmic tail in the inner leaflet of the proteoliposome, and anionic lipids influencing the bulk membrane properties other than electrostatic.

Local electrostatic effects may result in changes in local ion concentrations (charged peptide substrate, H$^+$, local decrease in pH). Electrostatic interactions involving anionic glycerophospholipids (which account for ~30% of cellular phospholipids) belong to the fundamental principles of biological interactions at membrane interfaces. A common characteristic of these nonspecific events is the requirement for a minimum interfacial concentration (~10 mol %) of the anionic phospholipid before an effect is observed (34). Because we see a striking effect already at a relatively low concentration of PA (5%), it is not clear whether this is the case in our system, where externally exposed anionic phospholipids could be responsible for the recruitment of the peptide substrate to the membrane. To avoid complications of this type, it
would be necessary to employ proteoliposomes with asymmetrically distributed lipids, restricting the anionic lipids to the inner side to mimic the topology of cell membranes. However, such systems have not yet been used for membrane protein studies, even though several promising attempts to produce asymmetrical liposomes have emerged (31, 32).

Under ionic strength conditions much lower than in our system (5–10 mM), the local pH near the surface of the membrane containing...
anionic phospholipids is 1.5–2 pH units lower than in bulk solution (33). Optimal pH for BACE activity is ~4.5, the activity steeply dropping above pH 5.5. If the local pH effects caused by the presence of PS/PA were the major mechanism for stimulating BACE activity in proteoliposomes, one would expect the decline in activity of reconstituted BACE to be less steep with increasing pH, which does not seem to be the case (supplemental Fig. S5).

Another way in which anionic lipids may be involved in stimulating BACE activity is a direct interaction of BACE with a negatively charged species in the outer leaflet, resulting in changes in BACE conformation, oligomeric state, or charge distribution. The only negatively charged species continuously and extensively present in the outer leaflet of the plasma membrane are charged glycosphingolipids (gangliosides and sulfatides). Role of gangliosides and sulfatides was not thoroughly investigated in this study. Small amounts of gangliosides (5% w/w, 2% mol/mol) used in mixtures with PC, PE, SM, and cholesterol, did not cause any observable change in activity. In addition, an interaction of the cytoplasmic tail with PS or PA (or PI, which, was not experimentally tested in this study) in the inner leaflet could as well affect the conformation of the protein. Anionic phospholipids have also been shown to strongly influence other bulk membrane properties, including lipid packing density and perturbing the bilayer structure (34). This phenomenon can account for facilitated penetration of the transmembrane domain, resulting in increased stability of BACE insertion, eventually affecting its activity.

As opposed to anionic phospholipids, glycosphingolipids mainly localize to the outer leaflet of the plasma membrane, and brain tissue in particular has been found to contain high amounts of neutral glycosphingolipids (cerebrosides). Brain cerebrosides contain mostly long saturated fatty acid chains (C22:0, C24:0, and also C24:1 (information from Avanti catalog)). The stimulatory effect of cerebrosides in proteoliposomes on BACE activity may be because of these long acyl chains or may be due to the presence of carbohydrate head groups that besides thickening the layer of perturbed water near the membrane interface may interact with the BACE ectodomain. Interestingly, cerebrosides also activate soluble BACE lacking the transmembrane and cytosolic part, and this further supports the possibility of the saccharide head group interacting with the BACE ectodomain. In addition, we demonstrate that also in cultured cells (glyco)sphingolipids affect the secretion of the Aβ peptide (see supplemental methods and Fig. S7). Recently, another study demonstrating a reduction of Aβ and βAPP secretion upon inhibition of glycosphingolipid biosynthesis was published (36).

Because our hypothesis assumes β-cleavage of APP to happen primarily in lipid rafts, we examined the possibility of BACE requiring the lo phase domain to assume a high activity state, by employing liposomes consisting of PC:SM:cholesterol (2:2:1 or 1:1:1 mol/mol),5 which has been shown to phase separate at relevant temperatures (35). Even though in GUVs BACE does not show a preference for the lo phase, the measured 15–20% seems to be a significant fraction when compared with other proteins that have been probed using the same technique. 25–30% of the glycosylphosphatidylinositol-anchored placental alkaline phosphatase, which has become a "prototype of a raft protein," and is recognized as a raft marker, partitions into lo phase in the same system (22), and also only 10–20% of a LAT-derived peptide was found to partition in the lo phase (37). On the other hand, syntaxins or bacteriophyosphatidylphosphatidylglycerol partition almost exclusively (95–99%) into ld.6 In addition, partitioning of BACE into the lo phase seems to be enhanced by cross-linking of the membrane components (we have used the pentavalent CTTB to cross-link GM1), offering potential means of regulating BACE-raft association in cells.

Even though neither cholesterol alone, nor the lo phase in the system per se can account for full activation, cholesterol levels clearly influence BACE activity. We show correlation between cholesterol levels and BACE activity in mixtures including PC, sphingolipids, and PE. Whereas a direct interaction of BACE with cholesterol (BACE was found to label strongly with photolabel cholesterol in the native environment of the cellular membranes, see supplementary results) cannot be excluded, it seems more probable that cholesterol content primarily affects the bulk properties of the liposomal membrane. In accord with this observation is the steep drop in activity of BACE in both asolectin (contain plant sterols) and total brain lipid proteoliposomes upon treatment with mβCD.

It should be noted that our experimental approach provides information on the mean specific activity of BACE in proteoliposomes of a particular average lipid composition. We cannot distinguish between a situation where a part of the pool of the reconstituted BACE would be completely inactive, and the rest present in a highly activated state (which may be the case in bilayers displaying lateral domain separation), from a situation where all BACE molecules would display the same activity. Despite this limitation, we have identified 3 classes of lipids affecting BACE activity in proteoliposomes: 1) anionic glycerophospholipids (whose stimulating effect is potentiated by PE); 2) (neutral) glycosphingolipids (cerebrosides); and 3) sterols (cholesterol).

All lipids are capable of affecting multiple properties of the membrane, and obviously, properties of individual lipid species are not combined by simple additive principles (note, for example, the effect of PE in combination with PS, but not alone, effect of cholesterol, or the effect of cerebrosides). Without understanding principles of lateral lipid organization, and without means to control vertical lipid organization in artificial systems, we cannot completely mimic the conditions in the native environment.

Nevertheless, using the soluble peptide substrate, we demonstrated that classical raft lipids, cerebrosides and particularly, cholesterol, promote activity of BACE in proteoliposomes. The role of raft lipids (and the role of phase-separated ld domains mimicking the raft environment) is likely to become more pronounced when BACE activity toward its natural substrate, the co-reconstituted full-length APP, is probed. To date, we have established that BACE cleaves the co-reconstituted APP in total brain lipid liposomes (supplemental Fig. S6) at the correct site, therefore our reconstituted system is relevant and should be well suited for testing the hypothesis of raft-mediated β-cleavage of the full-length APP.

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5 There was no significant difference when either POPC or DOPC was used.
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