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Imaging and Functional Analysis of γ -Secretase and Substrate in a Proteolipobead System with an Activity-Based Probe

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Abstract

Investigation of intramembranal protease catalysis demands the generation of intact biomembrane assemblies with structural integrity and lateral mobility. Here, we report the development of a microsphere supported-biomembrane platform enabling characterization of γ -secretase and substrate within proteolipobead assemblies via microscopy and flow cytometry. The active enzyme loading levels were tracked using an activity-based probe, with the biomembranes delineated by carbocyanine lipid reporters. Proteolipobeads formed from HeLa proteoliposomes gave rise to homogeneous distributions of active γ -secretase within supported biomembranes with native-like fluidity. The substrate loading into supported biomembranes was detergent-dependent, as evidenced by even colocalization of substrate and lipid tracers in confocal 3D imaging of individual proteolipobeads. Moreover, the loading level was tunable with bulk substrate concentration. γ -Secretase substrate cleavage and its inhibition within γ -secretase proteolipobeads were observed. This platform offers a means to visualize enzyme and substrate loading, activity, and inhibition in a controllable biomembrane microenvironment.

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

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Detailed description of the materials and methods (PDF)

The authors declare no competing financial interest.

Intramembrane proteases are extraordinary catalysts that hydrolyze the scissile bond of substrates within the lipid bilayer and play an essential role in the signaling paradigm of regulated intramembrane proteolysis.¹² γ -Secretase, a macro-molecular intramembranal protease that contains four subunits, Presenilin, Nicastrin, Aph1-, and Pen2,³ has emerged as an important therapeutic target for the treatment of Alzheimer's disease⁴ and cancer.⁵ Despite considerable methodology development, the current studies of γ -secretase in cell-free systems have been primarily carried out using either (1) membrane fragments,⁶ (2) detergent-solubilized membrane fragments,⁶ or (3) proteoliposomes.^{7,8} However, these assay formats preclude facile direct observation of biomembrane assemblies *in situ*, and it is difficult to directly assess enzyme and substrate loading levels. To probe membrane proteins in intact lipid bilayers, solid-supported biomembrane systems have emerged as a valuable means, enabling direct imaging of protein distributions and activity within biomembrane microenvironments of validated structural integrity and lateral mobility.^{9–11} Microsphere supported-biomembranes (also referred to as proteolipobeads or PLBs) are being explored for assays and functional studies of membrane proteins due to the potential for harnessing high-content multiplexing analyses using flow cytometry and quantitative fluorescence microscopy.^{9,12–14} In these systems each microsphere assembly can effectively function as a single cell-like assay element in high-content analyses, extending the investigation beyond the capabilities of proteoliposomes.¹⁵

In this study, we developed the construction of a microsphere-supported biomembrane platform for the investigation of γ -secretase and substrate in intact lipid bilayers. Furthermore, we analyzed the distribution and inhibition of γ -secretase in a single bead or microsphere within PLBs through an activity-based γ -secretase probe. Importantly, this platform, for the first time, allows us to investigate recombinant substrate loading and insertion into lipid bilayers. Taken together, this platform provides a novel way for investigation of γ -secretase function in intact lipid bilayers that offer critical insights into the mechanism of γ -secretase and substrate loading.

EXPERIMENTAL SECTION

Reagents

All lipids in this study were purchased from Avanti Polar Lipids, Inc. DiO (DiOC₁₈(3) (3,3'-dioctadecyloxycarbocyanine perchlorate)), DiD (DiIC₁₈(5) ((1,1'-Diocetadecyl-3,3,3',3'-Tetra-methylindodicarbocyanine Perchlorate)), streptavidin-FITC, and Alexa Fluor633-

streptavidin were obtained from Invitrogen (Molecular Probes). Silica microspheres of 5 μm nominal size were obtained from Bangs Laboratories, Inc. Anti-FLAG-FITC was obtained from Sigma-Aldrich. The AlphaLISA reagents were purchased from PerkinElmer Inc. (Waltham, MA).

Preparation of Liposomes

Medium-sized Unilamellar Vesicle (MUVs, 100 nm) were made from mixtures of lipids using extrusion. The liposome composition was L- α -phosphatidylcholine/ cholesterol/brain total lipid extract at a 56:10:34 mass ratio, with DiO of DiD added as a lipid tracer at a 1:500 or 1:250 molar ratio with respect to the mixture. Lipids were mixed as CHCl_3 solutions in a round-bottomed flask, dried as a thin film under reduced pressure in a rotary evaporator for 20 min, and evacuated under high vacuum for 2 h. The lipid film was resuspended in 5 mL of buffer B (20 mM Tris, 150 mM NaCl, 10 mM MgCl_2 , pH = 7.4). Then, the resuspension mixture was frozen at -80°C in liquid nitrogen and thawed at 65°C in a water bath, repeated 5 times. MUVs were prepared by 10 extrusion cycles with an extruder from Lipex Biomembranes Inc. by running multilamellar vesicles through two stacked 100 nm filters using a nitrogen gas pressure of 350–400 psi, which provided a homogeneous batch of liposomes.

Preparation of HeLa Proteolipobeads

HeLa cell membrane fragments were solubilized with 1% CHAPSO.⁶ The solubilized membrane fragments were mixed with the liposomes in successive aliquots to give specified mg protein/mg lipid ratios followed by dilution to give less than 0.01% final CHAPSO concentration. Debris and aggregates were removed by centrifugation to give a clear solution of DIO-doped HeLa proteoliposomes. HeLa proteoliposomes were fused with 5 μm nominal size silica microspheres (Bangs Laboratories) for 30 min at a ratio of greater than 10:1 lipid bilayer area to total microsphere surface area, followed by four wash steps to remove excess proteoliposomes or any γ -secretase containing debris or membrane fragments.

Flow Cytometry Analysis

Flow cytometry measurements were made using an Accuri C6 cytometer (Accuri Cytometers)) to obtain 15 000 PLB counts that were then gated on the prominent forward scatter feature of dispersed (single assembly) 5 μm nominal size silica microspheres, depicted in in the Supporting Information. This excluded most of the undesired multimicrosphere complexes (the arrow points to the PLB microsphere population from doublets) and highly fluorescent debris from the measurements. All flow cytometry runs contained control samples to measure relative mean channel fluorescence as absolute channel intensities varied after multiple manufacturer servicing visits. FlowJo 10.0.7 (Tree Star, Inc., Ashland, OR) was used to construct all 1D and 2D histograms and obtain the mean channel fluorescence (MCF, in arbitrary units (A.U.)), the mode, and the coefficient of variation (CV). In some cases, the x -axis of the cytometric histogram was plotted as a linear scale (versus the traditional log scale) to aid in closer visual examination of the statistical attributes (Figure 4d).

Confocal Microscopy

Confocal microscopy was used to image AlexaFluor 633 labeled streptavidin (AF633-SA) bound to the surface of the proteolipobead assemblies, revealing the distributions of γ -secretase:Cpd5:AF633-SA complexes. Samples were imaged using a Leica TCS SP2 AOBS confocal microscope system equipped with argon ion and HeNe lasers. A 63 $\times$ /1.4 NA oil immersion objective was used for all the images. Alexa Fluor 633 was excited using the 633 nm line of a He/Ne laser and images were taken with the detection window set between 645 and 750 nm. The DiO lipid probe (DiO, C₁₈(3)3,3'-dioctadecyloxacarbocyanine) was excited with the 488 nm laser line and detected between 500 and 550 nm. The DiD lipid probe (DiD, C₁₈(3)3,3'-dioctadecyloxacarbocyanine) was excited with the 633 nm laser line and detected between 650 and 750 nm. Sequential line-by-line scanning was used to eliminate crosstalk. The pinhole aperture was set at an Airy value of 1.0, which was equivalent to >500 nm vertical slice of the bead in each Z section. Samples were compared under the same detector and laser settings in adjacent wells sharing the same coverglass by employing 8-well Lab-Tek II no. 1.5 chambered coverglasses (Nunc, Thermo Fisher Scientific, Chicago, IL). In final image processing, all image adjustments were made identically to the entire set of images that were under comparison. PLBs that appeared to have compromised lipid coverage due to damage during handling or those found in multiparticle aggregates on the slide were not included in this image galleries displayed. For CLSM data, ImageJ64 was used to make summed Z-projections of the Z stacks to a single image and measure maximum intensity per PLB, the total integrated signal intensity per PLB. To measure lipid coverage and surface defects, each CLSM hemisphere of a PLB was projected to a plane and dark areas of missing DiO (or DiD) staining were measured and corrected for distortion effects on the area measurements considering the data as an azimuthal equidistant projection of a hemisphere (as outlined in the Supporting Information).

RESULTS AND DISCUSSION

Construction of Microsphere-Supported HeLa Membrane Protein Assemblies (Proteolipobeads)

There are two core materials for PLB construction: (1) CHAPSO-solubilized HeLa cell membrane fragments and (2) 100 nm diameter extruded liposomes doped with the lipid reporter probe DiO (DiO, C₁₈(3)3,3'-dioctadecyloxacarbocyanine). As depicted in the schematic flowchart of Figure 1a, mixing of these components yield proteoliposomes that are then fused with 5 μ m silica microspheres to give HeLa membrane protein proteolipobeads.¹⁴ To monitor the function and biomembrane distribution of γ -secretase, Compound **5** (Cpd5, Figure 1a,b),¹⁶¹⁷ an activity-based probe (ABP) that only binds the active form of γ -secretase, is utilized. The biotin-tethered Cpd5 probe thus provides a handle for measuring γ -secretase activity via modulation of biotin display (detectable using Alexa Fluor 633-streptavidin readout). Probe Cpd5 is bound to accessible and active γ -secretase in the lipid bilayer of proteolipobead assemblies, followed by the binding of AF633-SA to give a readout of the amount displayed at the PLB surface. It remains to be investigated whether opposite orientations of γ -secretase both bind to Cpd5. The distribution of a representative subset of γ -secretase:ABP complexes present in the lipid bilayer as assessed by Cpd5 biotin

displayed on the PLB surface (Figure 1a, left), whereas inhibition can be investigated by testing if a given candidate molecule blocks the binding of Cpd5, leading to a reduction or abolishment of ABP biotin display (Figure 1a, right).

γ -Secretase and Inhibitor Complex in PLBs under Flow Cytometric Detection

The lipobead format¹⁸ allows us to easily evaluate the multichannel scattering and fluorescence intensity histograms of thousands of microsphere assemblies via flow cytometry. This assay format yields pertinent mean channel fluorescence (MCF) values for the sample in each channel and furthermore enables the assessment of the biomembrane quality and relative loading levels of active enzyme and substrate in PLBs. Closer examination of the statistical attributes of the histograms including asymmetry, relative distribution widths, population comparison, and evidence for multimodal histograms (multiple overlapping populations) can be correlated to direct visualization of supported biomembranes via confocal fluorescence microscopy. The total and nonspecific binding of Cpd5 to γ -secretase in the proteolipobeads are defined in the absence of and presence of excess L-685,458¹⁹ (1 μ M), a parent compound of Cpd5. The specific binding, an indicator of γ -secretase activity, is calculated from the difference between the total and nonspecific binding. The active γ -secretase embodied in PLBs was analyzed by flow cytometry (Figure 1c). AF633-fluorescence per proteolipobead assembly in different conditions was shown in Figure 1c. The detection of active γ -secretase was assessed after assembling PLBs by mixing varying ratios of solubilized protein and lipid. Each histogram was obtained from 15 000 total PLB microsphere counts then gated by the forward scatter feature of dispersed 5 μ m microsphere assemblies, effectively excluding most of the undesired multimicrosphere complexes and any fluorescent debris from the measurements (see the Supporting Information, Figure S1). The back histogram (Figure 1c, 1-III) set was from 1:1 protein/lipid HeLa PLBs incubated with Cpd5 (–) or blocked by preincubation with 1 μ M L-685,458 (+) giving rise to ~34-fold MCF value. The middle histogram (Figure 1c, 1-II) set was from 10:1 protein/lipid HeLa PLBs gives rise to a ~20-fold MCF value difference. The front histogram (Figure 1c, 1-I) set was from 1:10 protein/lipid HeLa PLBs and showed there is no specific binding. Of note, the histograms displayed here are narrow and approximately symmetric, with small positive skewness and coefficient of variation (CV; (standard deviation/mean)) values at 110 or below with no evidence for multiple overlapping populations (unimodal distributions). These results indicate that this method enables us to begin to manipulate and also validate the relative amount of active enzyme in intact lipid bilayers, a pivotal experimental variable in further investigation of γ -secretase enzymology and critical in the design and construction of new assays.

To further characterize γ -secretase and lipids in PLBs, the DiO lipid fluorescence probe, a long-chain dialkylcarbocyanine, was incorporated into the beads for dual detection of the γ -secretase:Cpd5:AF633-SA and DiO-stained lipid (Figure 1d). Displayed in Figure 1d is the blocked binding of Cpd5 by preincubation with 1 μ M L-685,458, yielding population histogram (+), relative to the sample without added L-685,458 (population histogram (–)). These lipid bilayers are of similar high quality, given the DiO histogram symmetry (slight negative skewness), narrow distribution widths (CV values below 65), and unimodal nature.

In other words, no appreciable overlapping populations, an indicator of heterogeneity, were observed.

Examination of γ -Secretase Proteolipobeads via Confocal Microscopy

These microspheres also allow for the analysis of the 3D distribution of the ternary complex of γ -secretase:Cpd5:AF633-SA in lipid bilayers by confocal laser-scanning microscopy (CLSM). This technique complements flow cytometry, as information concerning the heterogeneity of the AF633-SA on the proteolipobead surface and the lipid coverage/distribution and their relative colocalization is directly visualized. Furthermore, confocal fluorescence recovery after photobleaching studies are used in tandem with 3D imaging are used to further characterize the biomembrane quality of HeLa PLB complexes by examining the DiO probe lateral mobility within the supported bilayers.^{13,20}

After forming the γ -secretase-Compound 5 (Cpd5) complexes, the biotin moiety of Cpd5 on the supported biomembrane surface was visualized by AlexaFluor-633 conjugated streptavidin (red, AF633-SA). A representative set of confocal images of HeLa γ -secretase proteolipobeads incubated with AF633-SA pertaining to the uninhibited case (Cpd5 only) is displayed in Figure 2a. CLSM of HeLa PLBs shown in Figure 2a; (top) in which the AF633-SA (red) and DiO lipid reporter (green) channel 3D intensity Z data is projected from a PLB hemisphere as a sum to a single image to highlight visual features of γ -secretase loading and biomembrane structure. The top images were obtained from the γ -secretase:Cpd5:AF633-SA XYZ data set, which indicate the distribution of active γ -secretase:Cpd5:AF633-SA complexes embedded in the supported bilayer accessible by Cpd5. The bottom images were obtained from the respective DiO lipid reporter XYZ data (green), showing the localization of the supported lipid bilayer positioned on the microsphere surface. Figure 2b displays the AF633-SA channel of γ -secretase proteolipobeads sample preincubated and “blocked” with L-685,458 (100 nM) followed by incubation with Cpd5 (10 nM), with the respective DiO-doped lipid channel. The top images were obtained from 3D reconstructions of the AF633-SA XYZ data set, which indicate the distribution of active γ -secretase. The bottom images were obtained from 3D reconstruction of the respective DiO XYZ data set. The blocked γ -secretase population (100 nM L-685,458 + Cpd5) exhibit an irregular surface coverage in the Alexa Fluor 633-SA channel; however, they have the similar levels of homogeneous biomembrane surface coverage indicated by the DiO fluorescence 3D reconstructions, suggesting that L-685,458 does not damage the lipid bilayer. Sparse and irregular surface coverage with highly fluorescent deposits is an indicator of nonspecific binding and aggregation of AF633-SA. We note that the AF633-SA aggregates are not appreciably correlated with regions of high DiO (defect) fluorescence in these images. Although current optical resolution does not allow for probing local conformation, this PLB system offer a valuable mean for investigating intramembranal enzymology.

Confocal FRAP was investigated in a random sampling of HeLa PLBs within these samples, and the majority of the regions studied showed DiO mobility, yielding an average mobile fraction of $\alpha = 0.87 \pm 0.02$ and an average diffusion coefficient of $D = 0.15 \pm 0.03 \mu\text{m}^2/\text{s}$ ($N = 5$). As a control we probed the DiO mobility of HeLa free lipobeads using the liposome starting material composition, yielding an average mobile fraction of $\alpha = 0.97 \pm 0.04$ and an

average diffusion coefficient of $D = 0.19 \pm 0.03 \mu\text{m}^2/\text{s}$ ($N = 5$). These studies indicate that fluid lipid bilayers are largely present in the HeLa proteolipobead assemblies with statistically insignificant differences ($p = 0.25$, calculated with D values) in DiO diffusivity relative to the control “membrane protein-free” lipobeads. We note that these DiO diffusivities are lower than those commonly found in unsupported bilayers, as expected;²¹ furthermore, the lipid formulation used will contain nanoscopic liquid order domains, increasing the lipid bilayer viscosity. Given that there is a statistically significant ($p = 0.003$, calculated with α values) lower mobile fraction in the HeLa PLBs, as some fraction of biomembrane heterogeneity that limits lateral diffusion in the form of defects in lipid coverage is present in some regions of these assemblies, as might be expected as the proteoliposomes used for fusion are derived from a mixture of CHAPSO-solubilized HeLa membrane proteins. Nevertheless, high levels of DiO fluorescence has been shown to delineate regions of intact cell membrane since excited state deactivation due to photoisomerization dramatically decreases the DiO quantum yield when in conformationally disordered microenvironments in bulk solution or adsorbed at interfaces.²²

A quantitative CLSM examination of the DiO-stained 3D lipid coverage of a larger number of individual beads in each case showed that the uninhibited case (Cpd5 only) displayed in Figure 2a exhibited a lipid surface coverage of $96.6 \pm 1.1\%$ ($N = 20$) and in the L-685,458 blocked case shown in Figure 2b the coverage was $97.1 \pm 1.1\%$ ($N = 20$), statistically insignificant differences ($p = 0.5$). Collectively, the highly similar distributions of DiO in the absence and presence of L-685,458 indicates the formation of homogeneously distributed lipid bilayers on the microsphere surface. These images are consistent with the lipobead motif where a single mobile lipid bilayer embedded with active γ -secretase resulting from fused proteoliposomes is supported on the silica microsphere, separated by a thin water layer from the SiO_2 surface. We do not see significant DiO CLSM signal heterogeneity that is consistent with appreciable mixed vesicle adsorption. Furthermore, these images illustrate that L-685,458 specifically blocks the interaction of γ -secretase and Cpd5 and does not perturb the structural integrity of the supported lipid bilayers in the proteolipobead assemblies. In addition, we also conducted nonspecific binding experiments with bare substrate beads without lipids present (data not shown). Under these conditions ($0.5 \mu\text{g}/\text{mL}$ AF633-SA), highly variable and irregular nonspecific binding and AF633-SA aggregation occurred at very low levels, undetectable under the same CLSM amplification settings.

Loading of SB4 Substrate into PLB Assemblies

Since *in vitro* γ -secretase activity assays were developed using recombinant substrate in the presence of CHAPSO, information about how the substrate loads into the membrane to meet γ -secretase for cleavage has been elusive due to technical challenges.⁶ Taking advantage of the proteolipobead system that allows us to visualize and quantify the substrate loading level into intact supported lipid bilayers, we investigated γ -secretase substrate SB4 insertion into membranes in PLBs. SB4 is biotinylated near the N-terminus and contains a FLAG epitope tag at the C-terminus (depicted schematically in Figure 3a). This substrate encompasses the transmembrane domain and C-terminal fragment of APP for γ -secretase processing.^{23–26} This substrate can be detected at both ends using streptavidin-fluorophore conjugates at the biotin site and Anti-FLAG-FITC at the C-terminus. In cleavage assays the detergent

CHAPSO is required for reconstitution of γ -secretase activity, with 0.25% found to be the most favorable concentration.^{6,23}

A schematic of the experimental design of the loading of the SB4 is shown in Figure 3a. Fluorescence intensity per bead histograms were obtained from flow cytometry of dispersed single bead complexes by gating the measurement to the forward scattering channel in the single particle region. Figure 3b shows the results of SB4 loading into PLBs, using loading concentrations of 0 μ M, 0.5 μ M, and 1 μ M SB4 in 0.25% CHAPSO. For reference a control sample is included where CHAPSO was not included during the incubation with 1 μ M SB4. The CHAPSO is then removed by successive washing, equilibration, and centrifugation steps, in contrast to a limitation of bulk proteoliposome assays where washing is not feasible. Anti-FLAG-FITC was used to detect the presence of SB4 in the bilayers, via binding to the FLAG epitope tag at the C-terminus, and a DiD lipid tracer was used to label the lipid bilayer (DiD, 1,1'-Diiododecyl-3,3,3',3'-tetramethylindocarbocyanine; emission max. ~670 nm). The mean channel fluorescence in the anti-FLAG-FITC goes up approximately linear with SB4 concentration, increasing from a MCF of 4851 at 0.5 μ M (Figure 3b, 3-II) to 9898 at 1 μ M SB4 (Figure 3b, 3-III). The distributions shown in the cytometric histograms are both narrow and symmetric, with similar standard deviation (SD) values of 5436 and 4885, respectively, with moderately low CV values indicative of homogeneous surface coverage. By comparison, the top histogram (Figure 3b, 3-IV) displays data from the control experiment where no additional CHAPSO was added to facilitate SB4 to PLB loading. The histogram is broad and heterogeneity is evident, with the appearance of being composed of multiple components with differing distributions (multimodal) and furthermore, the distribution SD value has gone up greater than 2-fold. In the control case where no SB4 was added, only low levels of anti-FLAG-FITC nonspecific binding to the PLBs were obtained, yielding a FITC channel MCF of 383.

These results correlate to confocal imaging studies of SB4 loading into PLBs under the same conditions. For the 0.25% CHAPSO loading case, a representative set of PLB CLSM images are shown in Figure 3c in which anti-FLAG-FITC ((SB4) green) and DiD lipid reporter (red) channel 3D intensity Z data from a hemisphere is projected as a sum to a single image to highlight visual features of loading and biomembrane structure. The top left 0.25% CHAPSO images were obtained from the anti-FLAG-FITC XYZ data set, which indicate the distribution of loaded SB4 (green). The bottom images were obtained from the respective DiD XYZ data that shows the localization of the supported lipid bilayer positioned on the microsphere surface (red). The 0.25% CHAPSO proteolipobeads displayed relatively uniform surface coverage in both the anti-FLAG-FITC and DiD channels, indicative of successful SB4 loading into the PLBs supported biomembranes. Images from the 0% CHAPSO SB4 loading experiment is shown in Figure 3c (bottom), from a representative set of microspheres from the case where CHAPSO was not present to facilitate SB4 loading. This surface coverage is highly heterogeneous with extensive aggregation in the anti-FLAG-FITC (SB4) channel (top) and the DiD intensity (bottom) is somewhat variable and dark in areas, indicative of losses in DiD staining consistent with compromised lipid bilayer structural integrity or possibly extraction of DiD with SB4 assemblies that interact with the supported bilayers. In some cases the substrate is found in regions distinct from the DiD reporter staining (uncolocalized), indicative of the substrate aggregated at the interface and

unable to diffuse into the supported lipid bilayer. The percent PLB surface colocalization of SB4 and DiD reporter was determined to be $96.6 \pm 1.1\%$ for the 0.25% CHAPSO sample, relative to $23.6 \pm 2.6\%$ when CHAPSO was not added to facilitate loading ($N = 20$ PLBs), a greater than 4-fold increase in colocalization. This finding, taken together with the homogeneous loading versus heterogeneous separation within the CLSM images is consistent with CHAPSO playing a key role in facilitating the delivery of substrate into the intact lipid bilayers, presumably aiding in overcoming substrate diffusion barriers that lead to even distributions within the lipid bilayer. The supported bilayers remained mostly intact in the 0.25% CHAPSO SB4 loading case with a percent DiD coverage of $96.6 \pm 1.1\%$, compared with $51.2 \pm 4.7\%$, in the 0% CHAPSO SB4 loading case ($N = 20$ PLBs).

Figure 3d are the 2D flow cytometric histograms obtained from the same samples as the CLSM. The 0.25% CHAPSO facilitated loading case resulted in narrow, unimodal histograms in both the anti-FLAG-FITC and DiD lipid reporter channels, with CV values below 60 for each channel. By contrast, in the SB4 loading sample where CHAPSO was not included, the 2D histogram exhibits a complex, multimodal distribution as seen in the adjunct histograms in both the anti-FLAG-FITC and DiD channels, with a greater than 2-fold increase in CV value in the anti-FLAG-FITC channel (59.5–141).

Given that SB4 substrate contains sequences designed for imaging at each end, the PLB format enables a powerful new route to be used to assess substrate MP orientation. Figure 3e shows the flow cytometric histograms resulting from SB4 orientation studies where streptavidin-FITC was used to detect biotin and anti-FLAG-FITC was used to localize the C-terminal FLAG tag. After loading at 0.25% CHAPSO, the uncorrected MCF value for the anti-FLAG-FITC binding to was ~2.5-fold greater than the MCF obtained from streptavidin-FITC bound at the SB4 biotinylation site. PLBs with without SB4 (negative control) yielded low background levels (MCF = 1004). The proteolipobead SB4 orientation assay gives an approximately 56% outward SB4 orientation (SB4 FLAG sequence oriented outward from the PLB), corrected for the degree of biotinylation of SB4 (~90%) and the measured F/P ratios of anti-FLAG-FITC (F/P = 4.4) and streptavidin-FITC (F/P = 2.4). In addition to enabling the characterization of the orientation relative to SB4 loading conditions (detergent, lipid composition, etc.), this method could provide a route to probing the rates of cleavage products released from PLBs using direct imaging or cytometry-based measurements. These findings, for the first time, provide direct evidence that the delivery of the substrate to the supported lipid bilayers is facilitated in the presence of detergent.

γ -Secretase Proteolipobeads Interfaced with Cleavage Assays

The ability to load γ -secretase and its substrates or effector proteins into well-characterized supported biomembranes via proteolipobead assemblies stands to add a new dimension in intramembrane protease target reagent delivery to the extensive range of assays. In order to further validate the HeLa PLB system, HeLa proteolipobeads (from Figure 1) was used to conduct a γ -secretase activity assays for A β 40 production from SB4 substrate delivered via 0.25% CHAPSO. For the case of HeLa PLBs created by forming 1:1 mg protein/mg lipid proteoliposomes, the cleaved product was detected by the AlphaLISA, and HeLa PLBs showed nearly the same percent specific activity as the HeLa proteoliposomes used to

construct the PLBs ($97\% \pm 8.2$ versus $100\% \pm 4.8$; normalized to the HeLa proteoliposomes ($N = 2$)). The lack of a statistically significant difference between the two samples indicates that the presence of the silica microsphere support in the HeLa PLBs did not perturb cleavage activity in the biomembranes.

Figure 4a shows the 2D histogram utilized to observe simultaneously the AF633-SA and DiO lipid reporter fluorescence distributions of the sample prior to loading into the cleavage assay. Since there was no Cpd5 added, the AF633-SA shows only the background signal histogram; however, the DiO distribution is narrow and symmetric with a slight negative skewness and a CV value of 35. This histogram is consistent with HeLa PLBs with high-quality supported biomembranes delivered to the assay. The results from the dosage-response AlphaLISA cleavage assay using this HeLa PLB sample are shown in Figure 4b with an IC_{50} value of 0.57 nM, consistent with earlier reports for L-685,458; however, in these case for the first time we have conducted the assay under defined and characterized conditions of biomembrane structure and substrate loading.

To characterize and validate the HeLa PLB sample added to the cleavage assay we also conducted CLSM and flow cytometry under L-685,458 blocking and Cpd5 incubation at concentrations of 0 nM and 100 nM of the inhibitor. Figure 4c displays a comparison of PLB images where the Alexa Fluor 633-SA channel 3D data is projected as a sum to a single image to highlight visual features of inhibition, at each indicated concentration with the same detector gain. The unblocked (0 nM L-685,458) 3D CLSM case displays an even distribution of intense Alexa Fluor 633-SA staining, indicative of high levels of the γ -secretase:Cpd5:AF633-SA complex distributed in the supported bilayer. In contrast, the 100 nM inhibited sample (blocked) shows only a weak yet mostly uniform intensity distribution punctuated in some fraction of the PLBs by the presence of highly fluorescent AF633-SA aggregates. In essence, the CLSM imagery reveals the PLB visual signature of the active enzyme distribution and nonspecific background achieved by L-685,458 blocking of the PLBs. We reiterate here that the bare silica microspheres do not appreciably adsorb Alexa Fluor 633-SA at 0.5 $\mu\text{g/mL}$.

These samples were studied under flow cytometry, and the imaging findings correlate with the statistical attributes of the histograms obtained from thousands of PLBs. Figure 4d displays the corresponding Alexa Fluor 633-SA channel flow cytometry histograms under L-685,458 incubation at concentrations of 0 nM and 100 nM, using a linear scale to highlight features of the distributions (in other cytometry histogram figures a log scale was used (Figures 1, 3, and 4a)). The arrows indicate to relative values of the mean channel fluorescence (MCF) relative to the mode of the distributions (mode, the intensity value that appears most often in the distribution). In the uninhibited case (0 nM L-685,458; top histogram) the distribution is symmetric with nearly identical mean and mode values, with a low skewness ($(\text{MCF}-\text{Mode})/\text{SD}$). In contrast, the 100 nM blocked case (100 nM L685,458; bottom histogram) give an asymmetric distribution with positive skewness (mean > mode; histograms tailing off to higher intensity) as an appreciable number of PLBs contain higher than average signal intensities due to the presence of the highly fluorescent aggregates, a signature of nonspecific binding.

CONCLUSIONS

Development of platforms that allow for investigation of intramembranal proteases in the intact biomembrane assemblies with structural integrity and lateral mobility under controlled conditions have been a formidable challenge. To attack this problem we have undertaken the reconstitution of γ -secretase into supported biomembranes via proteolipobeads and analyzed these assemblies with FACS and CLSM.

The tandem of FACS and CLSM allow one to examine the validity and quality of samples via flow histogram parameters and to distinguish the signature of inhibition and relevant γ -secretase specific phenomena from anomalous staining due to nonspecific binding and low biomembrane quality. These studies can lead to the ultimate development of a PLB fluorescence-activated cell sorting (FACS) criteria and insures that only the highest quality sorted subset of PLBs is loaded into a given γ -secretase assay in high-throughput format, in terms of enzyme and substrate loading, and also homogeneity and coverage of the supported biomembranes. Moreover, the CLSM structure and mobility studies aid in further quantifying the nature of the active enzyme distribution and lipid bilayer structural integrity for γ -secretase. Furthermore, the PLB method can be used as a platform to exert control over the loading of relative amounts of γ -secretase substrate into intact lipid bilayers proximal to the enzyme, another crucial experimental variable in investigations of γ -secretase enzymology and assay design. The determination of the exact amount of γ -secretase loaded in PLBs by flow cytometry might be feasible using Mean Equivalents of Soluble Fluorescence technology or biotin-based calibration. However, how microenvironment influences fluorescence quantum yield needs to be carefully investigated for accurate measurements. In essence, the solid support of the PLB system provides a stable platform to manipulate biomembrane composition by allowing the lipid bilayer to remain positioned when hydrophobic substrates are being delivered. This is in contrast to unsupported systems such as proteoliposomes or giant unilamellar vesicles (GUVs), where the addition of 1 μ M SB4 in 0.25% CHAPSO would be predicted to have strongly perturbative effects on biomembrane structure and composition.

The proteolipobead research direction represents a novel approach to expanding *in vitro* models of γ -secretase and other intramembrane proteases, using flow cytometry and fluorescence microscopy, which are powerful and well-developed techniques. Bringing these technologies to bear on intramembrane proteases such as γ -secretase in intact lipids in PLBs increases the scope of feasible *in vitro* assays and opens up new avenues of investigation. The PLB assay system has enabled the characterization and manipulation of the relative biomembrane loaded levels of active enzyme and substrate into lipid bilayers. Importantly, this system unprecedentedly demonstrated how detergent affects the insertion of γ -secretase substrate into biomembranes and provided a means to determine substrate orientation. The PLB system can extended from crude to highly purified membrane protein preparations where the lipid composition can be tuned to include complex membrane phase-separated domain structures that can be confocal imaged to allow phase partitioning studies of the enzyme and substrates. Currently, the PLB platform helps to uncover details that are missed in bulk solubilized membrane studies and will provide a new platform for the study protein–protein interactions. The PLB system enables studies of the mechanism of γ -secretase

modulators and γ -secretase inhibitors, studied under controlled conditions of protein and lipid composition in supported biomembranes, with the inclusion of cholesterol-rich microdomains thought to be linked to Alzheimer's disease.²⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Construction of γ -secretase proteolipobeads: (a) Schematic representation of γ -secretase proteolipobead assay. (b) The structure of compound **5** (Cpd5) contains an inhibitory pharmacophore and a biotin linker. (c) Flow histograms of AlexaFluor-633 conjugated streptavidin binding to γ -secretase:cpd5 complexes within HeLa proteolipobeads at different levels of enzyme loading. Each histogram was obtained from 15 000 total PLB microsphere counts then gated by the forward scatter feature of dispersed 5 μm microsphere assemblies comparing blocked sample preincubated with L-685,458, blocking binding of Cpd5, and biotin display. The top histogram set (1-III) was from 1:1 protein/lipid HeLa PLBs incubated with Cpd5 (–) or blocked by preincubation with 1 μM L685,458 (+) giving rise to ~34-fold mean channel fluorescence (MCF) value decrease. The middle histogram set (1-II) was from 10:1 protein/lipid HeLa PLBs incubated with Cpd5 (–) or blocked by preincubation with 1

μM L685,458 (+) giving rise to a ~20-fold MCF value decrease. The bottom histogram set (1-I) was from 1:10 protein/lipid HeLa PLBs incubated with Cpd5 (–) or blocked by preincubation with 1 μM L685,458 (+) giving rise to a ~0-fold MCF value decrease. (d) 2D flow cytometric histograms of AlexaFluor-633 conjugated streptavidin binding to DiO lipid tracer doped HeLa proteolipobeads for the 1:1 protein/lipid HeLa PLBs case shown above in part c. Population (–) was from HeLa PLBs incubated with Cpd5 as compared with a parallel γ -secretase proteolipobeads sample preincubated with L685,458 (population (+)), blocking binding of Cpd5, and biotin display. The y-axis contains the adjunct histograms from the DiO lipid reporter channel.

Figure 2.

Confocal microscopy of HeLa proteolipobeads. Confocal microscopy of representative γ -secretase proteolipobeads containing γ -secretase:Cpd5:AF633-SA complexes and DiO lipid reporter fluorophores. After forming the γ -secretase-Compound 5 (Cpd5) complexes, the biotin moiety of Cpd5 on the supported biomembrane surface was visualized by AlexaFluor-633 conjugated streptavidin (red, AF633-SA). CLSM of HeLa PLBs in which AF633-SA (red) and DiO lipid reporter (green) channel 3D intensity Z data is projected from a PLB hemisphere as a sum to a single image to highlight visual features of γ -secretase loading and biomembrane structure. (a) The images were obtained from the γ -secretase:Cpd5:AF633-SA XYZ data set, which indicate the distribution of active γ -secretase:Cpd5:AF633-SA complexes (red) embedded in the supported bilayer (green). The bottom images were obtained from the respective DiO lipid reporter XYZ data (green). (b) AF633:AF633-SA channel of γ -secretase proteolipobeads sample preincubated and "blocked" with L-685,458 (100 nM) followed by incubation with Cpd5 (10 nM), with the respective DiO doped lipid channel.

Figure 3.

SB4 substrate loading into PLB assemblies. (a) Schematic flowchart of the loading of SB4. (b) Flow cytometric histogram results of SB4 loading into PLBs, using loading concentrations of 0 μ M, 0.5 μ M, and 1 μ M SB4 in 0.25% CHAPSO. The anti-FLAG-FITC increases from a MCF of 4851 (blue histogram, 3-II) at 0.5 μ M to 9898 (dark green histogram, 3-III) at 1 μ M SB4. The control case where no SB4 was added yielded a MCF of 383 (red histogram, 3-I). Histogram 3-IV is from the 1 μ M SB4 loading experiment without CHAPSO. (c) CLSM of PLBs with 0.25% CHAPSO SB4 loading. Anti-FLAG-FITC (green) and DiD lipid reporter (red) channel 3D intensity Z data from a hemisphere is projected as a sum to a single image to highlight visual features of loading and biomembrane structure. The 0.25% CHAPSO top images were obtained from the anti-FLAG-FITC XYZ data set, which indicate the distribution of loaded SB4 (green). The 0.25% CHAPSO bottom images were obtained from the respective DiD XYZ data (red). The top 0% CHAPSO images were

obtained from the anti-FLAG-FITC XYZ data set, which indicate the distribution of loaded SB4 (green). The bottom images were obtained from the respective DiD XYZ data (red). (d) 2D flow cytometric histograms obtained from the same samples as the CLSM, for the 0.25% CHAPSO case (top) and 0% CHAPSO case (bottom). (e) Results from studies of SB4 substrate orientation within PLB assemblies. On the left are the flow cytometric histogram results of SB4 loading into PLBs (1 μ M SB4 in 0.25% CHAPSO). The anti-FLAG-FITC detection yielded an MCF of 24,185 (blue histogram, 3-VI) and the streptavidin-FITC MCF value was 9306 (red histogram, 3-VII). The control case where no SB4 was added yielded a MCF of 1004 (green histogram, 3-V).

Figure 4.

Interfacing HeLa γ -secretase proteolipobeads with high-throughput cleavage assays. (a) The 2D flow cytometric histogram utilized to observe simultaneously the AF633-SA and DiO lipid reporter fluorescence distributions of the sample prior to loading into the cleavage assay. No Cpd5 was added, and the AF633-SA shows only the background signal histogram; however, the DiO distribution is narrow and symmetric with a slight negative skewness and a CV value of 35. (b) The results from a dosage-response PLB cleavage assays using this HeLa PLB set. The solid lines in the graph are from a 4-parameter sigmoidal dosage response fit curve that yielded an IC_{50} value of 0.57 nM. (c) PLB CLSM of L685,458 incubation at concentrations of 0 nM and 100 nM prior to Cpd5 binding, with the Alexa Fluor 633-SA channel 3D data projected as a sum to a single image, to highlight visual features of γ -secretase:Cpd5:AF633-SA complex distribution and its inhibition (same detector gain). (d) Corresponding Alexa Fluor 633-SA channel flow cytometry histograms under L685,458 incubation at concentrations of 0 nM (4-I) and 100 nM (4-II), using a linear scale. The arrows indicate to relative values of the mean channel fluorescence (MCF) relative to the mode of the distributions.