Biochimica et Biophysica Acta 1778 (2008) 1356–1364

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Role of ceramide in membrane protein organization investigated by combined AFM and FCS

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ARTICLE INFO

Article history:
Received 30 November 2007
Received in revised form 29 January 2008
Accepted 13 February 2008
Available online 29 February 2008

Keywords:
Ceramide
Raft
Liquid-ordered
Membrane protein
GPI anchor
Fluorescence correlations spectroscopy
Atomic force microscopy

ABSTRACT

Ceramide-induced alterations in the lateral organization of membrane proteins can be involved in several biological contexts, ranging from apoptosis to viral infections. In order to investigate such alterations in a simple model, we used a combined approach of atomic force microscopy, scanning fluorescence correlation spectroscopy and confocal fluorescence imaging to study the partitioning of different membrane components in sphingomyelin/dioleoyl-phosphatidylcholine/cholesterol/ceramide supported bilayers. Such model membranes exhibit coexistence of liquid-disordered, liquid-ordered (raft-like) and ceramide-rich lipid phases. Our results show that components with poor affinity toward the liquid-ordered phase, such as several fluorescent lipid analogues or the synaptic protein Synaptobrevin 2, are excluded from ceramide-rich domains. Conversely, we show for the first time that the raft-associated protein placental alkaline phosphatase (GPI-PLAP) and the ganglioside G_{M1} are enriched in such domains, while exhibiting a strong decrease in lateral diffusion. Analogue modulation of the local concentration and dynamics of membrane proteins/receptors by ceramide can be of crucial importance for the biological functions of cell membranes.

1. Introduction

In the last decade, experimental evidence has led to the hypothesis that cell membrane constituents might be organized in small lipid/protein domains enriched in sphingomyelin (SM) and cholesterol, known as "rafts" [1,2]. The physical state of these domains is considered to be similar to a liquid-ordered (L_o) phase, structurally and dynamically distinct from the rest of the lipid bilayer which is, in turn, assumed to be in a liquid-disordered phase (L_d) [3]. Lipid microdomains are thought to be involved in various biological processes such as protein and lipid sorting for cell–cell communication or intracellular signaling cascades [4].

Several studies have identified a novel membrane domain based on the sphingolipid ceramide, which plays an important role in a multitude of cellular processes ranging from differentiation to immune response and apoptosis [5–7]. Because of its unusual biophysical properties [8,5], ceramide molecules display a tendency to separate from the rest of the membrane, forming gel-phase, ceramide-rich domains [5,6], both in cells [9] and in model membranes [10–12]. In particular, such domains were also observed when ceramide was added or produced in bilayers that showed coexisting L_d/L_o, raft-like phase separation [13–15]. It is important to stress that, at least in the context of simple model membrane systems, it is possible to distinguish "lipid rafts", which are L_o domains enriched in SM and cholesterol, from "ceramide-rich domains", highly ordered gel domains that probably exclude cholesterol [16,17].

The interplay between rafts and ceramide domains is thought to be involved in the internalization of viruses and parasites and in the induction of apoptosis [18]. Ceramide-rich platforms may act in these situations as sorting locations for membrane receptors, inhibitors and other membrane components involved in signaling [19]. For example, ceramide-rich domains seem to recruit the receptors mediating the internalization of *Neisseria gonorrhoeae* [20]. Similar receptor clustering and trapping in ceramide-rich domains is also suggested by experiments performed with Fcγ receptor II [21], CD95 and CD40 [22,23]. Nevertheless, since the ceramide phase is characterized by tight packing of lipids and high structural order, it seems counter-intuitive that certain membrane proteins would partition strongly into these rigid domains [15]. Until now, no biophysical study concerning the affinity of membrane proteins or sphingolipids (other than SM and ceramide) for the ceramide-rich domains has been reported.

In order to address this question, we used a combined approach of atomic force microscopy (AFM), fluorescence imaging, and scanning fluorescence correlation spectroscopy (scanning FCS) to study the partitioning of membrane components into different lipid phases. Specifically, we produced supported bilayers showing a coexistence of L_d phase, L_o raft-like phase and ceramide-rich gel phase. We investigated the lateral organization of L_d-associated membrane components — i.e. fluorescent lipid analogues and the synaptic membrane protein Synaptobrevin 2 — and two typical raft-associated membrane components —

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doi:10.1016/j.bbamem.2008.02.008

i.e. the glycosylphosphatidylinositol anchored protein placental alkaline phosphatase (GPI-PLAP), and cholera toxin (CT*x*-B) bound to G_{M1}. In order to obtain quantitative data about the partitioning of fluorescent molecules into the different lipid phases, we used a novel technique based on scanning FCS. FCS was already successfully employed to study dynamics in lipid membranes [24], and calibration-free techniques like 2-focus FCS [25] and *z*-scan FCS [26] were shown to provide precise and reliable diffusion coefficients. Use of scanning FCS, differently from the above described FCS approaches, makes it possible to obtain reliable data even in the case of very slow diffusion and strong bleaching of the membrane components which are present in the highly viscous regions of the membrane, such as the ceramide-rich domains. Furthermore, FCS in general allows us to neglect changes in brightness of the fluorophores in different lipid environments that arise because of selective excitation, quenching or environmental sensitivity.

Our results show that membrane components with a low capacity to partition into the raft-like L_o phase tend to be completely excluded from the tightly packed ceramide domains. On the contrary, GPI-PLAP and CT*x*-B, which usually show a remarkably high degree of partitioning into the L_o phase[1], seem to be concentrated in the ceramide-rich phase. Nevertheless, the affinity for the L_o phase is not by itself sufficient for the inclusion in ceramide domains, as exemplified by the case of the novel fluorescent free cholesterol analogue BODIPY-FChol. Furthermore, while the addition of ceramide does not appreciably change the dynamic properties of the membrane components in the L_d and L_o phases, very slow ($<<0.1\ \mu m^2/s$) diffusion is observed for the proteins enriched in the ceramide phase. These findings strongly support the hypothesis that ceramide domains may act in vivo as protein/lipid platforms that recruit or exclude specific membrane components (e.g. from small transient rafts), clustering them stably together and effectively slowing their in-plane diffusion.

2. Materials and methods

2.1. Chemicals

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (dioleoyl-phosphatidylcholine; DOPC), *N*-stearoyl-D-*erythro*-sphingosylphosphorylcholine (*N*-stearoyl sphingomyelin; SM), *N*-stearoyl-D-*erythro*-sphingosine (C18:0 ceramide, C18-Cer), Galβ1-3GalNAcβ1-4(NeuAcα2-3) Galβ1-4Glcβ1-1′-Cer (G_{M1} ganglioside, G_{M1}) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. BODIPY free cholesterol analogue 23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-24-norchol-5-en-3β-ol (BODIPY-FChol) was synthesized as described for "compound 2" in [28]. Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD-C18, DiD), cholesteryl 4,4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-dodecanoate (cholesteryl ester BODIPY FL C_{12}, BODIPY-CholE) and Alexa Fluor 488 cholera toxin subunit B (CT*x*-B) were from Invitrogen (Eugene, OR). Optical Adhesive 88, used to glue the mica on coverslips, was purchased from Norland Products (Cranbury, NJ). Alkaline phosphatase from human placental tissue (PLAP), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) and sodium cholate were purchased from Sigma. Before use, PLAP was purified and labelled with NHS-rhodamine (Pierce, Rockford, IL) using a protocol modified from [29] and [30]. In particular, high-resolution Superdex200 10/300GL (GE-Healthcare) columns were used instead of Sephacryl S200 and, after labelling, a Nap-5 (GE-Healthcare) and an additional Superdex columns were used to exclude free dye and protein dimers.

Recombinant protein Synaptobrevin 2 (residues 1–117C) was expressed, purified and labeled with Cy5 maleimide (Amersham Biosciences) as described in [31] and [32].Three different buffers were used for sample preparation and imaging: buffer A (3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 150 mM NaCl, pH 7.2); buffer B (3 mM $CaCl_2$, 150 mM NaCl, 10 mM Hepes, 3 mM NaN_3, pH 7.4); and buffer C (150 mM NaCl, 10 mM Hepes, 3 mM NaN_3, pH 7.4). All buffers were filtered through a 0.2-μm filter (Nalgene, Rochester, NY) prior to use.

2.2. Supported lipid bilayers (SLBs)

Planar bilayers were prepared based on the procedure described by Chiantia et al. [33]. Briefly, lipids and fluorescent lipid analogues were mixed in organic solvents in different proportions. The lipid composition was DOPC:SM:Cer:Cholesterol 1:0.7:0.3:0.67 molar ratio. In other terms, the concentrations of cholesterol and Cer were 25 and ~10 mol% of the lipid mixture, respectively. The concentration of fluorescent lipid analogues was always 0.1 or 0.01 mol%, for imaging and FCS, respectively. After solvent evaporation, the lipid film thus obtained was slowly rehydrated using buffer A at 10 mg/mL lipid concentration and resuspended by vigorous vortexing. After sonication of the suspension at 60 °C, a small aliquot was diluted in buffer B and deposited on a ~10-μm thick, freshly cleaved mica, glued to a glass coverslip. The coverslip was sealed in the temperature-controlled Biocell stage (JPK Instruments, Berlin, Germany), transferred to the microscope and incubated at 55 °C for 5 min. The sample was rinsed at the same temperature at least 10 times with buffer C and then allowed to cool to 25 °C. Samples containing Cer were generally unstable after ~3 h. For this reason, all the measurements were performed within before ~1 h after the cooling phase.

2.3. Protein-containing SLBs

In the case of membranes containing rhodamine-labeled PLAP, the liposomes were used to produce proteoliposomes before deposition onto mica, using a modification of the procedure described by Kahya et al. [29] In particular, the protein and 100 nm extruded liposomes (without ceramide) were mixed in buffer A to final concentrations of 40 μg/mL and 2.5 mg/mL, respectively, in the presence of 1.8% CHAPS. The liposomes' extrusion was performed twice: i) before adding CHAPS and ii) before adding the protein. The initial protein:lipid ratio was ~1:2500. After 24 h of dialysis against buffer A using a 50 kDa SpectraPro membrane (Spectrum, Breda, Netherlands), a small aliquot was diluted 10 times in buffer B, briefly sonicated with ceramide-containing liposomes in the desired proportions at 50 °C, and deposited onto mica as described above for the normal, protein-free, liposomes. The final protein:lipid ratio was then ~1:8000. The activity of the reconstituted protein was checked by enzymatic digestion of Sigma Fast *p*-nitrophenyl phosphate (Sigma) in the solution above the SLB.

SLBs containing Synaptobrevin 2 were prepared from proteoliposomes as described by Bacia et al. [31]. In particular, the protein and 100 nm extruded liposomes (without ceramide) were mixed in buffer A with ~1:2500 molar ratio, in the presence of 1.2% sodium cholate. The liposomes' extrusion was performed twice: i) before adding sodium cholate and ii) before adding the protein. After 24 h of dialysis against buffer A using a 50 kDa SpectraPro membrane (Spectrum, Breda, Netherlands), a small aliquot was diluted 10 times in buffer B, briefly sonicated with ceramide-containing liposomes in the desired proportions at 50 °C, and deposited onto mica as described above for the normal, protein-free liposomes. The final protein:lipid ratio was ~1:8500. The activity of the protein incorporated in the SLB was checked by specific binding to a fluorescent soluble syntaxing fragment (AA 183–262) [31].

In the case of samples stained with Alexa488 CT*x*-B, the SLBs contained 0.03% G_{M1}. The toxin (10 mg/mL) was added and washed away after 2 min incubation.

2.4. Optical setup

Confocal imaging and scanning FCS measurements were performed on an laser scanning microscope (LSM) Meta 510 system (Carl Zeiss, Jena, Germany) using a 40× NA 1.2 UV-VIS-IR C Apochromat water-immersion objective and a home-built detection unit at the fiber output channel. An appropriate band-pass filter was used behind a collimating achromat to reject the residual laser and background light. Another achromat (LINOS Photonics, Goettingen, Germany) with a shorter focal length was used to image the internal pinhole onto the aperture of the fiber connected to the avalanche photo diode (APD, PerkinElmer, Boston, MA). The photon arrival times were recorded in the photon mode of the hardware correlator Flex 02-01D (Correlator.com, Bridgewater, NJ). All filters and dichroic mirrors were purchased from AHF Analyse Technik, Tuebingen, Germany. The movement of the detection volume was controlled directly with the Zeiss LSM operation software.

2.5. Scanning FCS

Data were acquired by scanning repeatedly the focal volume in a linear fashion in the membrane. Line scans of ca. 5-μm length were chosen such that all three phases were scanned through. Intensity traces of parts of the scans corresponding to only one phase were correlated scan by scan. To account for photo bleaching, correlation curves $G_i(\tau)$ were multiplied by the ratio between the average intensity of the corresponding scan I_i and the initial intensity I_0: $\tilde{G}_i(\tau)=G_i(\tau)\cdot I/I_0$. Since the concentrations c_i are inversely proportional to amplitudes of the correlation curves (i.e. $G_i(0)\sim 1/c_i$) and directly proportional to the intensities ($I_i\sim c_i$), $\tilde{G}_i(\tau)$ is in this way scaled back to the initial correlation curve $G_0(\tau)$. The average of the rescaled correlation curves was fitted with a flow-diffusion model. Since the residence time of the scanned detection volume is much shorter than the diffusion time, the diffusional part of the correlation curves was very small. Therefore diffusion coefficients could be fixed to approximate value. By using the known velocity of the detection volume, the fit provided directly the size of the detection volume w_0 and, more importantly, the fluorophore concentration *c*.

Knowing the concentration of the fluorophores in the different phases (c_{Lo}, c_{Ld}, and c_{Cer}), we defined a "normalized partition" P_X for the phase X as:

$$P_X = \frac{c_X}{c_{Lo} + c_{Ld} + c_{Cer}} \quad (1)$$

In the case of samples without ceramide, P_X is simply:

$$P_X = \frac{c_X}{c_{Lo} + c_{Ld}} \quad (2)$$

[1] Compared to other lipid analogues and membrane proteins like SNAREs and Bacteriorhodopsin [27].