

Review

Lysenin: A sphingomyelin specific pore-forming toxin

Hidehiko Shogomori^a, Toshihide Kobayashi^{a,b,c,*}^a *Supra-Biomolecular System Research Group, RIKEN (Institute of Physical and Chemical Research) Frontier Research System, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan*^b *Lipid Biology Laboratory, RIKEN, 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan*^c *Inserm UMR 870, INRA U1235, INSA-Lyon, University Lyon 1 and Hospices Civils de Lyon, 69621 Villeurbanne, France*

Received 11 July 2007; received in revised form 8 August 2007; accepted 5 September 2007

Available online 15 September 2007

Abstract

Sphingomyelin is a major sphingolipid in mammalian cells. Recent results indicate that sphingomyelin is a reservoir of lipid second messengers, ceramide and sphingosine-1-phosphate. Sphingomyelin is also a major component of sphingolipid and cholesterol-rich membrane domains (lipid rafts). Lysenin is a pore-forming toxin that specifically binds sphingomyelin. The binding of lysenin to sphingomyelin is dependent on the membrane distribution of the lipid, i.e. the toxin selectively binds sphingomyelin clusters. Development of a non-toxic lysenin mutant revealed the spatial and functional heterogeneity of sphingolipid-rich membrane domains.

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Keywords: Lipid domain; Lipid rafts; Cholesterol; Glycolipids

1. Introduction

The presence of sphingomyelin is reported only in eukaryotic cells. Sphingomyelin is reported in the nematode, *Caenorhabditis elegans* [1]. In contrast, yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* do not contain sphingomyelin. Instead, these organisms have inositolphosphorylceramide and phosphoethanolamine ceramide, respectively [2,3]. In mammalian cells, sphingomyelin comprises 10–15% of the total phospholipids. Even higher levels of sphingomyelin are found in erythrocytes, ocular lenses, peripheral nerve tissue and brain. Within the cell, sphingomyelin is reported to be most abundant in the plasma membrane, especially the outer leaflet. Sphingomyelin metabolites play important roles as second messengers in signal transduction events during development and differentiation [4].

Sphingomyelin is also a major component of sphingolipid/cholesterol-rich membrane domains, called lipid rafts [5–7]. Although lipid rafts are proposed to be involved in various biological phenomena, the organization of lipid rafts is still a

matter of debate. Sphingomyelin-specific probes are a powerful tool to study the organization and biological function of this lipid.

Several pore-forming toxins have been reported to interact with sphingomyelin. The cytotoxicity of equinatoxin II from sea anemone *Actinia equina* is inhibited by the addition of sphingomyelin-containing membranes [8]. However, this toxin interacts with phosphatidylcholine and phosphatidylglycerol under appropriate conditions [9,10]. Sticholysin I and II from *Stichodactyla helianthus* also prefer sphingomyelin-containing membranes [11] and *Vibrio cholerae* cytolysin requires both sphingomyelin and cholesterol [12] for membrane insertion. Eiseniapore from the earthworm *Eisenia foetida* induces lysis of liposomes containing sphingomyelin or galactosylceramide [13]. Pleurotolysin is a novel sphingomyelin-specific two-component cytolysin from the mushroom *Pleurotus ostreatus* [14]. Lysenin is a sphingomyelin-specific pore-forming toxin (for review [15–18]). Recent characterization of this protein revealed that lysenin is a useful probe to study the organization of sphingomyelin in both model- and bio-membranes.

2. Lysenin is a sphingomyelin-specific pore-forming toxin

Lysenin is a 297 amino acid protein isolated from the coelomic fluid of the earthworm *Eisenia foetida* [19]. Lysenin

* Corresponding author. Lipid Biology Laboratory, RIKEN, 2-1 Hirosawa, Wako Saitama 351-0199. Tel.: +81 48 467 9534; fax: +81 48 467 9535.

E-mail address: kobayasi@riken.jp (T. Kobayashi).

induces hemolysis and has cytotoxicity to vertebrate spermatozoa and amphibian larvae as well as cultured mammalian cells [20–23]. A unique feature of lysenin is its specific binding to sphingomyelin. The following evidence supports the specific binding of lysenin to sphingomyelin:

- (1) Lysenin-induced hemolysis is specifically inhibited by sphingomyelin-containing liposomes [20].
- (2) In solid phase binding assay, lysenin selectively binds to sphingomyelin [20,24–26].
- (3) Lysenin specifically lyses sphingomyelin-containing liposomes [20].
- (4) Sphingomyelinase treatment of cultured cells abolishes the binding of lysenin [25].

In order to estimate the size of the pore formed by lysenin, hemolysis of sheep erythrocytes was measured in the presence of various carbohydrates and polymers that are known to inhibit hemolysis in a pore size-dependent manner [27]. Lysenin-induced hemolysis was inhibited by neither sucrose (effective molecular diameter; 0.9 nm) nor raffinose (1.2–1.4 nm), whereas dextran 4 (3–3.5 nm) and PEG 4000 (4 nm) significantly inhibited hemolysis [24]. These results suggest that the diameter of the pore induced by lysenin is ca. 3 nm. The ultrastructure of the lysenin-treated sphingomyelin-containing liposomes was examined by negative staining electron microscopy [24]. When sphingomyelin/cholesterol liposomes were incubated with lysenin, honeycomb-like regular hexagonal structures accumulated. The diameter of the hexagonal unit was 10–12 nm and there are pore-like structures of 3–5 nm diameter inside the hexagonal units (Fig. 1). Recent planar lipid bilayer experiments indicate that lysenin forms a voltage-dependent large conductance channel in a sphingomyelin-dependent manner [28,29].

The apparent molecular weight of lysenin was determined to be 41,000 by SDS-PAGE. When lysenin is added to sphingomyelin-containing liposomes, a 41-kDa band decreases and a new band with a molecular weight greater than 250,000 appears [24]. This indicates the formation of SDS-resistant lysenin oligomers in the presence of sphingomyelin. Lysenin contains 6 tryptophan residues. When lysenin was incubated with sphingomyelin-containing liposomes, the tryptophan fluorescence increased and the wavelength of maximum emission undergoes a blue shift from 332.8 to 330.3 nm, suggesting the migration of the tryptophan residues of lysenin to a less polar environment in the presence of sphingomyelin. When lysenin was incubated with sphingomyelins with different hydrocarbon chains at 37 °C, oligomerization was observed irrespective of the hydrocarbon chain of sphingomyelin. In contrast, lysenin oligomerized at 4 °C only when it was incubated with sphingomyelin containing unsaturated fatty acids. In contrast to oligomerization, the binding of lysenin to sphingomyelin was not significantly affected by the fatty acid composition of sphingomyelin. These results suggest that oligomerization but not binding is influenced by the fluidity of sphingomyelin.

Together with two additional proteins in coelomic fluid, referred to as lysenin-related protein 1 (LRP-1, lysenin 2) and

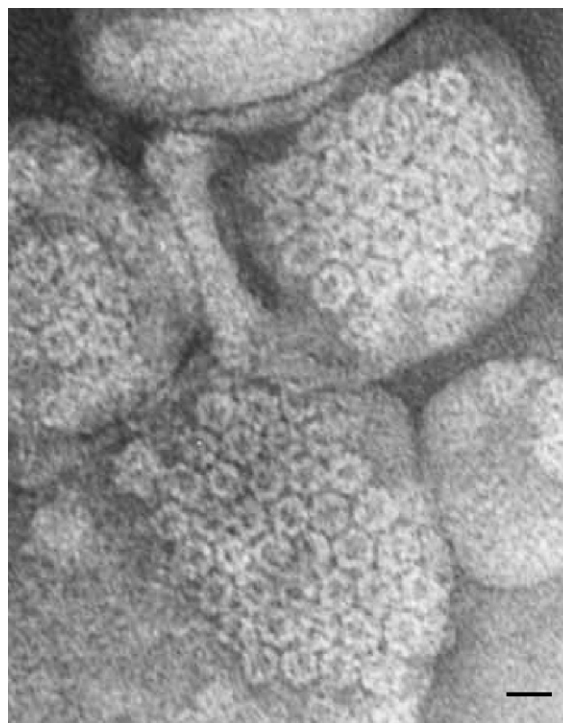


Fig. 1. Lysenin produces honeycomb structures in sphingomyelin-containing membranes. Brain sphingomyelin/cholesterol (1:1) liposomes (1 mmol/l lipids) were incubated with 400 µg/ml lysenin at 37 °C for 30 min. The mixture was fixed with 2.5% glutaraldehyde for 1 h at 37 °C, washed with phosphate-buffered saline, stained with 4% aqueous uranyl acetate and observed under a transmission electron microscope. Bar, 20 µm [16].

LRP-2 (lysenin 3), lysenin comprises a family of proteins sharing sequences of high homology [15,19]. The amino acids coded by LRP-1 cDNA are 76% identical to those of lysenin cDNA (89% for LRP-2 cDNA). The cDNA sequence of LRP-2 is identical to that of fetidin. The binding specificity and hemolytic activity of LRP-1 and LRP-2 were studied together with lysenin, using maltose-binding protein-tagged recombinant proteins [25]. LRP-2 specifically bound sphingomyelin and induced hemolysis in the same manner as lysenin. In contrast, the binding and hemolytic activities of LRP-1 were 10 times less than those of lysenin and LRP-2. Lysenin and LRP-2 share 30 common sites of aromatic amino acids. Among them, only one position, phenylalanine 210, is substituted for isoleucine in LRP-1. The activity of LRP-1 was dramatically increased by introducing a single amino acid substitution of isoleucine 210 to phenylalanine, suggesting the importance of this aromatic amino acid in the activity of lysenin and the LRPs. The importance of aromatic amino acids is further indicated by a systematic tryptophan to alanine mutation of lysenin. Among the 6 tryptophan residues of lysenin, five are conserved in LRP-1 and LRP-2. Using maltose-binding protein tagged lysenin, it was shown that the conserved tryptophans, but not the nonconserved one, were required both in the binding to sphingomyelin and the hemolytic activity of lysenin. Recently it was shown that substitution of tryptophan 20 by alanine was devoid of lytic activity, but retained binding activity to sphingomyelin in histidine-tagged lysenin [29]. Tryptophan 20 is a conserved amino acid among

lysenin and the LRP. This discrepancy between the two lysenins is explained by the different tag proteins (maltose binding protein vs. six histidine residues) used in the experiments [29].

3. Glycolipids inhibit lysenin binding to sphingomyelin, whereas cholesterol facilitates oligomerization of lysenin without affecting lipid binding

Recent results have indicated that the lipid environment affects the binding of lysenin to sphingomyelin. Epithelial cells contain two distinct plasma membranes; apical domains confront the external lumen whereas basolateral membranes face the underlying cell layer [30,31]. Each plasma membrane has a specialized function and contains a different set of lipids and proteins. Apical membranes are characterized by the enrichment of glycolipids [32]. In cultured kidney epithelial cells, MDCK II, the development of polarity is dependent on cell density. Lysenin binds to the apical surface of MDCK II cells when the cell density is low and thus cells are not polarized. In contrast, lysenin does not bind to the apical surface of highly polarized cells [33]. Selective recognition of basolateral surface by lysenin was confirmed by adding lysenin from the apical and basolateral sides of fully polarized MDCK cells. Cells were highly sensitive to lysenin when the toxin was added from the basolateral side, whereas cells were resistant to apically added toxin [33]. Sphingomyelin comprises 19% of the total phospholipids in the apical and 26.4% in the basolateral membranes [34]. A model membrane study showed that as little as 5% of sphingomyelin in the membrane is sufficient for lysenin to bind liposomes. These results indicate that the difference of sphingomyelin content between the apical and the basolateral membranes does not explain the different sensitivity of these membranes to lysenin. The role of glycolipids on the inhibition of lysenin binding to sphingomyelin was demonstrated using a melanoma mutant cell line. GM95 is a mouse melanoma mutant defective in ceramide glucosyltransferase, which catalyzes the first step of glycosphingolipid synthesis [35]. Thus GM95 is glycolipid deficient. Lysenin binds to GM95 but not to its parent cell, MEB4. Consistent with this observation, MEB4 was resistant but GM95 was sensitive to lysenin. Although MEB4 contains less sphingomyelin than GM95, the sensitivity of lysenin was not altered even after adjustment of the sphingomyelin content in these two cells by means of metabolic inhibitors [33]. These results suggest that glycolipids are inhibitory in the binding of lysenin to sphingomyelin.

The inhibitory role of glycolipids on the binding of lysenin to sphingomyelin was also examined in model membranes [33]. The binding of the toxin to the membranes was monitored by measuring the fluorescence resonance energy transfer (FRET) between the tryptophan residues of lysenin and the pyrene-labeled sphingomyelin incorporated into the membrane. Energy transfer was observed when lysenin was incubated with sphingomyelin/dioleoylphosphatidylcholine (C18:1 PC) membranes. The addition of galactosylceramide to these liposomes decreased the efficiency of FRET, indicating that glycolipid inhibits the binding of lysenin to sphingomyelin-containing

membranes [33]. The observed inhibitory effect was not restricted to galactosylceramide. A similar inhibitory effect was observed when GM1 (Gal β 1,3GalNAc β 1,4(NeuAc α 2,3)Gal β 1,4Glc β 1,1'-ceramide) or GM2 (GalNAc β 1,4(NeuAc α 2,3)Gal β 1,4Glc β 1,1'-ceramide) was added to the sphingomyelin-containing membranes [26]. The inhibition of binding of lysenin to sphingomyelin was also observed when C18:1 PC was replaced with dipalmitoylphosphatidylcholine (C16:0 PC). It is reported that sphingomyelin is immiscible with C18:1 PC [36]. In contrast, sphingomyelin and C16:0 PC are completely miscible [37]. This was confirmed in giant liposomes (GUVs) containing the fluorescent markers DiI C18 (1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate), which favors the solid phase, and BODIPY-C12-PC (2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine), which favors the fluid phase [38]. A clearly evident coexistence of the sphingomyelin-rich ordered phase and the C18:1 PC-rich fluid phase was observed in sphingomyelin/C18:1 PC vesicles. In contrast, in GUVs of sphingomyelin/C16:0 PC, uniform fluorescence of DiI C18 was observed. Lysenin preferentially bound to sphingomyelin/C18:1 PC GUVs (Fig. 2). These results suggest that lysenin recognizes sphingomyelin only when the lipid forms clusters. Isothermal titration calorimetry revealed that the stoichiometry of sphingomyelin and lysenin is 5.04, indicating one lysenin molecule binds 5 sphingomyelin molecules. These results show that the organization of sphingomyelin is different between MEB4 and GM95. These results also indicate that the apical and basolateral membranes of MDCK cells display altered sphingomyelin organization.

It has been shown that sphingomyelin/cholesterol liposomes are 10,000 times more effective than sphingomyelin liposomes in inhibiting lysenin-induced hemolysis [20]. Surface plasmon resonance measurements reveal that the dissociation constant of the binding of lysenin to sphingomyelin is not significantly altered by the presence of cholesterol in the membrane. The lack of the effect of cholesterol on the binding of lysenin to sphingomyelin was confirmed by isothermal titration calorimetry. The presence of cholesterol in the sphingomyelin membrane did not significantly alter the stoichiometry or thermodynamic parameters of sphingomyelin–lysenin complex formation [39]. The binding of lysenin to sphingomyelin/C18:1 PC and sphingomyelin/C18:1 PC/cholesterol was measured directly by separating membrane-bound and free lysenin. Cholesterol did not alter the amounts of membrane-bound lysenin [39]. These results together indicate that, unlike glycolipids, cholesterol does not affect the binding of lysenin to sphingomyelin-containing membranes.

Although cholesterol does not affect the binding of lysenin, cholesterol does facilitate the oligomerization of lysenin. Oligomerization of lysenin is also dependent on the sphingomyelin/lysenin ratio. When sphingomyelin/C18:1 PC liposomes were incubated with lysenin, the majority of the protein was oligomerized when the sphingomyelin/lysenin ratio was less than 240. In contrast, oligomerization was facilitated by sphingomyelin/C18:1PC/cholesterol liposomes irrespective of the sphingomyelin/lysenin ratio. The spectroscopic properties

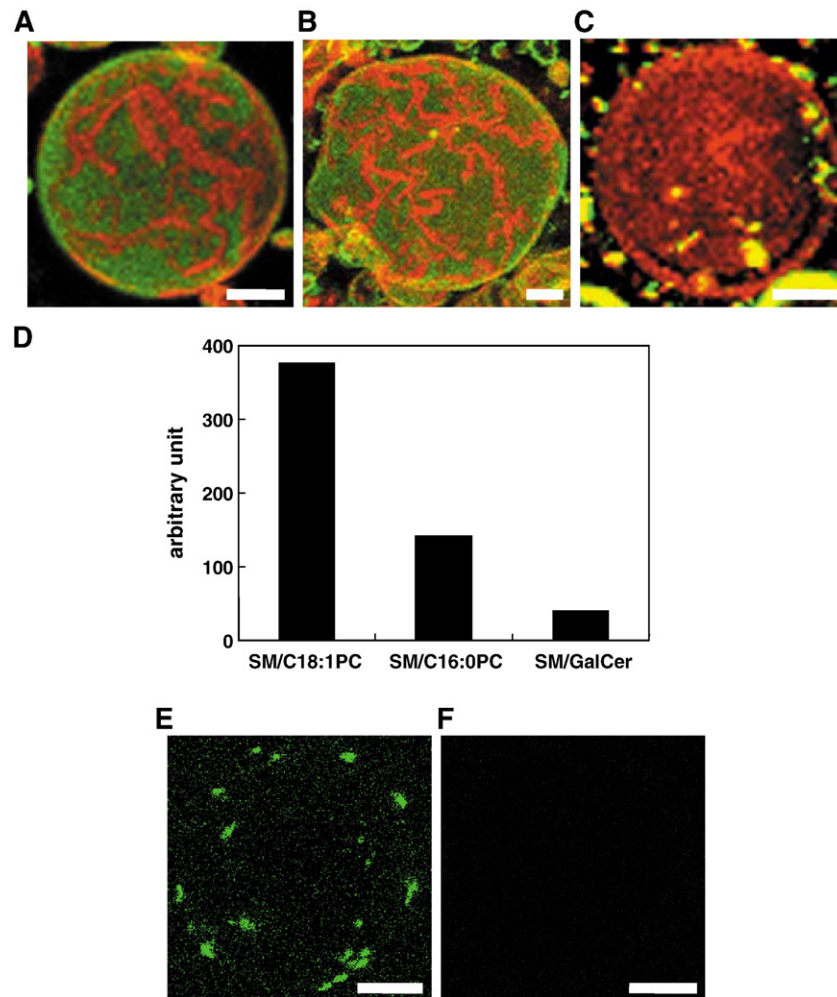


Fig. 2. Local density of sphingomyelin influences the binding of lysenin to sphingomyelin in model membranes. (A–C) GUVs composed of sphingomyelin/C18:1 PC (molar ratio 7:3) containing 7 mol% C16:0 phosphatidylglycerol and 3 mol% C12:0 phosphatidylglycerol (A and B) and sphingomyelin/C16:0 PC (molar ratio 1:1) containing 10 mol% C16:0 phosphatidylglycerol (C) labeled with 0.1% DiI C18 (red) and 0.1% BODIPY-C12-PC (green). Color merged images are shown. Bar, 2 μ m. (D) Binding of lysenin to sphingomyelin/C18:1 PC, sphingomyelin/C16:0 PC and sphingomyelin/galactosylceramide (molar ratio 3:7). (E and F) GUVs of sphingomyelin/C18:1 PC and sphingomyelin/C16:0 PC (molar ratio 3:7) were incubated with His-Venus lysenin. Bar, 5 μ m [33].

of lysenin oligomers monitored by tryptophan fluorescence and circular dichroism were not significantly changed by the presence of cholesterol [39].

Both monomer and oligomer lysenin associate with sphingomyelin-containing membranes. However, whereas the lysenin monomer is able to transfer to other membranes such as red blood cells, the lysenin oligomer does not transfer from one membrane to the other. In hemolysis inhibition experiments, the presence of cholesterol facilitates the oligomerization of lysenin and thus dramatically decreases the number of monomers responsible for hemolysis.

The above results indicate that lysenin binds sphingomyelin when the lipid form clusters. The presence of glycolipids hinders the formation of the sphingomyelin cluster and thus inhibits the binding of lysenin. Lysenin binds to sphingomyelin as a monomer. When sphingomyelin/lysenin ratio is lower than ca. 500, lysenin efficiently oligomerizes, perhaps because of the increased collision of monomers. Apparently, the role of cholesterol is to facilitate the collision of lysenin monomers. Since

cholesterol does not affect the binding of lysenin, cholesterol does not significantly alter the membrane distribution of sphingomyelin under our experimental conditions. Our results indicate the lack of lysenin binding does not mean a lack of sphingomyelin in the membrane.

4. Non-toxic lysenin reveals the heterogeneity of sphingolipid-rich membrane domains

One drawback of using full-length lysenin in cell biology is its toxicity. For the purpose of obtaining non-toxic lysenin, sphingomyelin-binding activity and toxicity of a series of deletion mutants of recombinant lysenin were recently examined [26]. Whereas the deletion of C-terminal amino acids diminished the recognition of sphingomyelin by lysenin, lysenin was able to bind sphingomyelin even after the removal of N-terminal amino acids. This is consistent with the recent observation that the N-terminus of lysenin has sequence homology to other pore-forming toxins [40]. It is noteworthy that all of the deletion

mutants lost their hemolytic activity. The minimal fragment that could bind sphingomyelin contains amino acids 161–297 of lysenin. This minimal peptide was named NT-Lys (non-toxic lysenin). NT-Lys retains its binding specificity to sphingomyelin. Whereas GST (glutathione-S-transferase)-lysenin oligomerizes in the presence of sphingomyelin-containing liposomes, GST-NT-Lys does not form oligomers under the same condition, suggesting that the oligomerization of the toxin is important for the toxicity of lysenin. The kinetic parameters of GST-NT-Lys binding to sphingomyelin were determined using surface plasmon resonance and compared with those of native lysenin. GST-NT-Lys and native lysenin exhibited comparable on-rate of binding to sphingomyelin ($k_{\text{on}} = 6.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for GST-NT-Lys and $3.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for native lysenin). In contrast, dissociation of GST-NT-Lys is 100 times faster than that of native lysenin ($k_{\text{off}} = 1.2 \times 10^{-2} \text{ s}^{-1}$ for GST-NT-Lys and $1.7 \times 10^{-4} \text{ s}^{-1}$ for native lysenin). This gives a 36-fold difference in the overall K_D ($K_D = 1.9 \times 10^{-7} \text{ M}$ for GST-NT-Lys and

$5.3 \times 10^{-9} \text{ M}$ for native lysenin). These results suggest that oligomerization of lysenin stabilizes the binding of the protein to sphingomyelin-containing membranes. Because of the low affinity, a relatively high concentration (ca. 50 $\mu\text{g/ml}$) of NT-Lys is required to label cells.

Lipid rafts are defined as sphingolipid- and cholesterol-rich membrane domains. Cholera toxin B-subunit (CTxB), which binds ganglioside GM1, has long been employed as a marker of lipid rafts. Using CTxB and NT-Lys, two sphingolipid-rich membrane domains were compared in Jurkat T cells [26]. When plasma membranes of living Jurkat cells were doubly labeled with CTxB and His-monomeric Venus GFP tagged NT-Lys (HmV-NT-Lys), cells were evenly stained, indicating that the size of the lipid domains are below the resolution of fluorescence microscopy. The distribution of GM1 and sphingomyelin was further examined on fixed two-dimensional sheets of plasma membrane ripped off from cells directly onto EM grids. Cells were doubly labeled with HmV-NT-Lys and

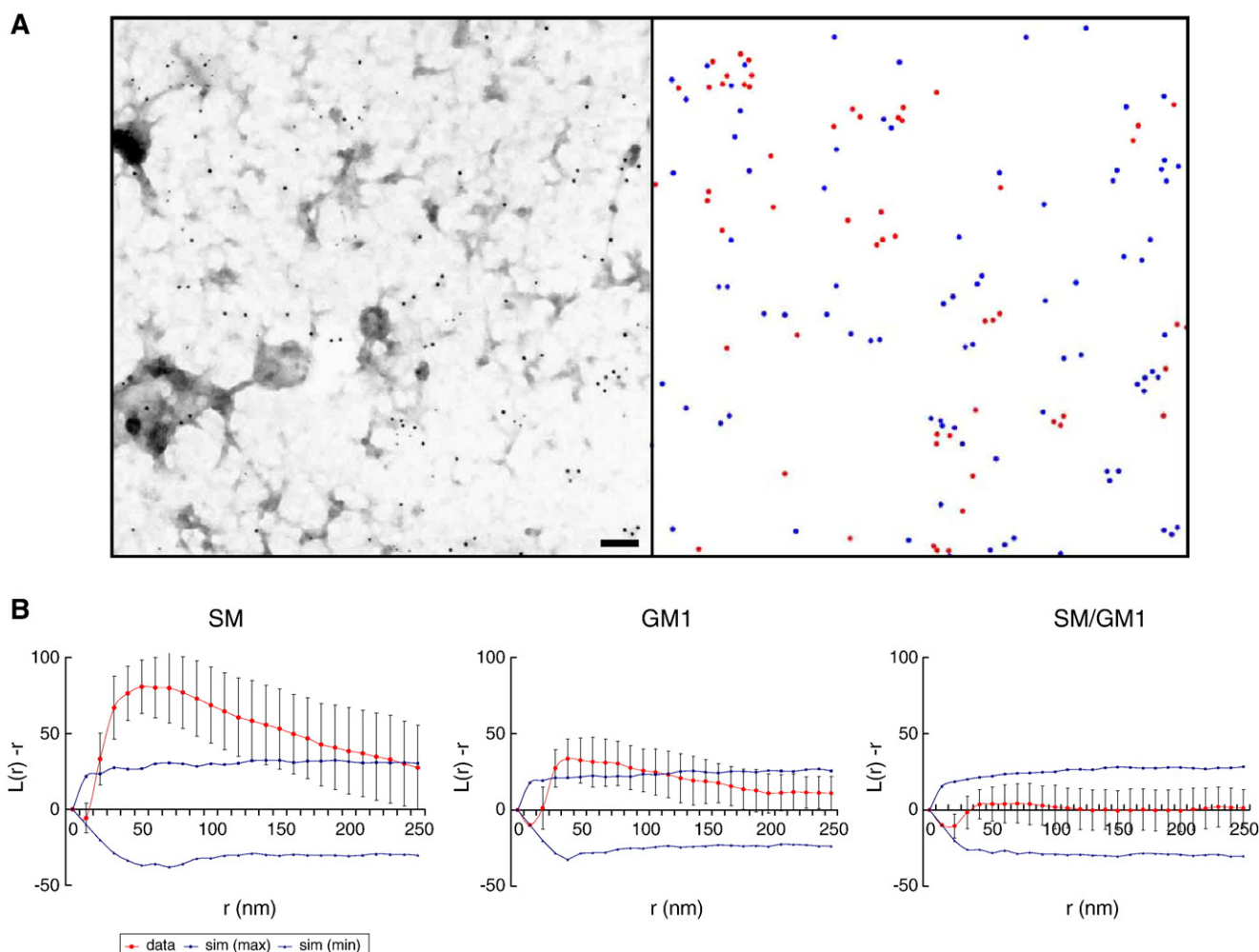


Fig. 3. Distribution of sphingomyelin-rich and GM1-rich membrane domains in two-dimensional sheets of plasma membrane from Jurkat cells. (A) Cells were labeled with HmV-NT-Lys and biotinylated CTxB at 4 °C. After fixation, the cells were further labeled with anti-GFP antibody followed by the incubation with anti-IgG-5 nm gold and anti-biotin-10 nm gold. The distribution of gold particles on the plasma membrane was examined under electron microscope after ripping off the membrane. Bar, 100 nm. Right panel, distribution of sphingomyelin (5 nm) indicates in red whereas the distribution of GM1 (10 nm gold) is in blue. (B) Analysis of the distribution of sphingomyelin-rich domain and GM1-rich domains using Ripley's K-function. Both sphingomyelin-rich and GM1-rich domains form clusters. Pairwise values for sphingomyelin and GM1 fall within blue lines that represent the range of values expected for pairs of different particles whose distribution is random [26].

biotinylated CTxB at low temperature. Both sphingomyelin and GM1 were distributed over the entire membrane. The gold patterns were further analyzed by using Ripley's K-function. Ripley's K-function evaluates all interparticle distances over the study area and compares the observed distribution of samples with that of complete spatial randomness [41,42]. The analysis indicates that both sphingomyelin and GM1 form domains with a radius of 60–80 nm. However, the colocalization of sphingomyelin-rich domains and GM1-rich domains is not significant (Fig. 3). These results thus indicate that plasma membrane sphingomyelin-rich domains are spatially distinct from ganglioside GM1-rich membrane domains.

In the same manner as T cell receptor activation and the cross-linking of GM1, the cross-linking of sphingomyelin by NT-Lys induces calcium influx and ERK phosphorylation in Jurkat cells [26]. However, unlike CD3 or GM1, the cross-linking of sphingomyelin did not induce significant protein tyrosine phosphorylation. These results suggest that sphingomyelin provides a functional signal cascade platform that is distinct from those provided by TCR and GM1. This idea is supported by the observation that sphingomyelinase treatment of Jurkat cells abolished LPA-mediated but not TCR-dependent signal transduction. These results suggest that LPA-mediated signal transduction is functionally related to sphingomyelin-rich membrane domains and is distinct from signal transduction pathways mediated by anti-CD3 antibody or the cross-linking of GM1.

5. Perspectives

Lysenin and non-toxic lysenin have made it possible to study the distribution of sphingomyelin-rich membrane domains in model and biological membranes. One potential problem of using proteins as a lipid probe is the difference in the size of lipids and proteins. Indeed, the molecular weight of lysenin is 30 times greater than that of phospholipids. It is also possible that the binding of lysenin alters the behavior of sphingomyelin. Nevertheless, an appropriate use of lysenin will help uncover the organization of sphingomyelin in various systems. A limited number of toxins are known to selectively bind specific lipids. Cholera toxin is known to bind GM1 as described above. Shiga toxin binds glycolipid Gb3 [43] and aerolysin recognizes GPI-anchored proteins [44]. Cinnamycin and duramycin are cyclic peptides that specifically bind ethanolamine phospholipids [45–47]. Perfringolysin O [48] and streptolysin O [49] bind cholesterol. The further development and detailed characterization of lipid-binding toxins will provide useful tools for the visualization of lipids in the future.

Acknowledgments

T. K. was supported by Grants from the Ministry of Education, Science, Sports and Culture of Japan, Grants from RIKEN Frontier Research System, Bioarchitect Research Project and Chemical Biology Research Project of RIKEN, RIKEN Presidential Research Grant for Intersystem Collaboration and International HDL Research Award Program.

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