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Septin Interferes with the Temperature-Dependent Domain Formation and Disappearance of Lipid Bilayer Membranes

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KEYWORDS

Membrane domain formation, Partitioning of lipid membrane, Septin, Supported lipid bilayer.

ABSTRACT

Domain formation or compartmentalization in a lipid bilayer membrane has been thought to take place dynamically in cell membranes and play important roles in the spatiotemporal regulation of their physiological functions. In addition, the membrane skeleton, which is a protein assembly beneath the cell membrane, also regulates the properties, as well as the morphology, of membranes, due to its role as a diffusion barrier against constitutive molecules of the membrane or as a scaffold for physiological reactions. Therefore, it is important to study the relationship between lipid bilayer membranes and proteins that form the membrane skeleton. Among cytoskeletal systems, septin is unique because it forms arrays on liposomes that contain phosphoinositides, and this property is thought to contribute to the formation of the annulus in sperm flagellum. In this study, a supported lipid bilayer (SLB) was used to investigate the effect of septin on lipid bilayers because SLBs rather than liposomes are suitable for observation of the membrane domains formed. We found that SLBs containing phosphatidylinositol (PI) reversibly form domains by decreasing the temperature, and that septin affects both the formation and the disappearance of the cooling-induced domain. Septin inhibits the growth of cooling-induced domains during decreases in temperature, and inhibits the dispersion and the disappearance of those domains during increases in temperature. These results indicate that septin complexes, *i.e.* filaments or oligomers assembling on the surface of lipid bilayer membranes, can regulate the dynamics of domain formation, via their behavior as an anchor for PI molecules.

1. Introduction

1.1. Membrane Domains

The basic structure of the cell membrane is a lipid bilayer, and a number of proteins indispensable for the activities of cells are incorporated. In the fluid mosaic model that was considered initially, lipids and proteins were thought to diffuse freely within the membrane. But currently, the model wherein specific lipids and proteins assemble and form membrane domains, where the compositions and characteristics are different from the surrounding areas, is accepted. [1–4] As the representative examples, lipid rafts and caveolae structures, micro-domains rich in cholesterol, sphingomyelin or glycolipids, are membrane domains that have attracted much attention. Caveolae are relatively stable structures formed by the interaction between a membrane and caveolin, a membrane-lining protein. In addition, recent studies revealed the physiological significance of membrane domains for the interactions between proteins and lipid membranes, the formation of protein assemblies such as membrane skeletons, the localization of proteins at membrane sites where the proteins to exhibit the function, and the membrane vesicle traffic in cells. [5–7] However, the mechanism of formation and maintenance of membrane domains where proteins are involved is still unclear.

1.2. Supported Lipid Bilayers (SLBs)

The supported lipid bilayer (SLB) was used as a model of a lipid bilayer membrane in this study. [8–13] The basic structure of the SLB is identical to that of membrane vesicles that have been used in many studies as the simplest model of the lipid bilayer membrane. The SLB exists at the interface between a solid substrate and an aqueous solution, and has a flat shape and maintains lateral diffusion properties, because about 1 nm thick water layer exists between the SLB and the

solid substrate. Therefore, SLBs have the ability to cause phase separation in the same way as other lipid membranes, and that ability can be regulated by varying the conditions. SLBs have recently been noted because they are suitable for analyzing the dynamics of target membrane-constituting molecules and investigating the mechanisms of reactions that occur in the membrane. Utilizing SLBs, the phase separation concerning membrane domains or rafts and the relationship between rafts and membrane proteins have been studied.[14–18] To observe SLBs, fluorescence microscopy, including confocal or total internal reflection microscopy, and atomic force microscopy (AFM) are frequently used.

It is already known that domains are inducible in SLBs prepared from synthetic lipids, *e.g.*, dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC), by changing the temperature. In those SLBs, the two phospholipids are completely mixed at 13℃ or higher temperatures, whereas the domains emerge at around 12.5℃ and they grow in size with decreasing temperature.[8] In this study, we also found that SLBs made from phosphatidylcholine (PC) and phosphatidylinositol (PI), phospholipids obtained from natural sources but not synthetic ones, show the formation and disappearance of PI-free domains by changing the temperature.

1.3. Septin, A Possible Regulator of the Membrane Domain

Septins can be purified from diverse types of eukaryotic cells, and they are regarded as the fourth cytoskeleton in addition to actin, microtubules and intermediate filaments.[19–21] Septin filaments polymerized can further assemble into diverse higher-order structures *in vivo* and *in vitro*, such as linear/circular/spiral bundles or gauze-like arrays, and have been implicated in a variety of organization processes of biological membranes.[20, 22–29] However, the detailed mechanism that septins exert the physiological roles remains unknown.

Septin is unique compared to the other cytoskeletons because it interacts directly with negatively charged lipid membranes via a cluster of basic amino acid residues at its surface.[30] Septins forming arrays beneath the cell membrane are estimated to give rigidity to the cell cortex and to become a scaffold for membrane proteins, although the details are unclear.[22] In addition, our previous *in vitro* study revealed that, when septin is added to giant liposomes, 1) the polymerization of septin filaments is stimulated, 2) the septin filaments spontaneously assemble into two-dimensional arrays on the liposome surface (Figure 1), and 3) liposomes show robust tubulation as a result of the array formation.[30] For these reactions to occur, an acidic phospholipid, such as PI or PI-4,5-bisphosphate (PIP_2), has to be contained in the membrane. The two-dimensional arrays of septin formed on the membrane surface are also considered to serve as a diffusion barrier.

The effects of the interaction with a lipid membrane on the nature of septin have been reported by studies using PIP_2-containing SLBs or lipid monolayers that were prepared on a flat substrate.[31,32] On the other hand, the effect of the interaction with septin on the nature of a lipid membrane, especially the partitioning of the membrane such as the domain formation, has not yet been revealed, except that an *in silico* study suggested that septin filaments work as a membrane diffusion barrier.[33] In *in vitro* experimental studies, mainly the morphogenesis of membranes has been studied regarding the impact of septin.[30,34] In this study, in order to use SLBs placed at low temperatures as a model for membrane domains, we prepared SLBs from PC and PI, and then cooled them to cause the domain formation. By adding septin to these SLBs before and after the membrane domains are formed, we investigated whether septin can regulate the membrane domain formation and affect the fluidity of the lipid bilayer membrane.

2. Materials and Methods

2.1. Regents

Phosphatidylinositol (PI) isolated from bovine liver was purchased from Avanti Polar Lipids (Alabaster, AL USA). Fluorescent-labeled annexin V and fluorescent-labeled lipid, N-(Texas Red sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE), were purchased from Takara Bio (Kusatsu, Shiga, Japan). A fluorescent-labeled lipid, 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC) was purchased from Invitrogen (Waltham, MA USA). Phosphatidylcholine (PC) and phosphatidylglycerol (PG) isolated from egg yolk, phosphatidylserine (PS) isolated from bovine brain, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine (DMPS) were purchased from Sigma (St. Louis, MO USA). The protein inhibitor cocktail (complete and EDTA-free tablet) used for preparing the septin fraction was purchased from Roche Diagnostics (Tokyo, Japan). Other chemicals were of analytical grade, and were used without further purification.

2.2. Supported Lipid Bilayers (SLBs)

In this study, SLBs were prepared by the method of vesicle fusion, because that method has the advantage of producing uniform SLBs throughout the solid substrates, regardless of their three-dimensional shape and size. [8–13, 35] Each vesicle solution required to prepare SLBs was obtained as follows. [36] Phospholipids were dissolved in chloroform/methanol solution (98:2, vol/vol) at concentrations of 100 μM for fluorescent-labeled lipids (BODIPY-PC and TR-DHPE) and 10 mM for the other lipids. They were stored away from light at 4℃ until use. A total 300 μL 10

mM lipids (PC and PI in this study usually) and 30 μL 100 μM BODIPY-PC or TR-DHPE were mixed into pre-washed vial tubes (10 mL). The lipid composition of SLBs was PC, PI, and BODIPY-PC (1:1:0.002, mol/mol), or PC, PI, and TR-DHPE (4:1:0.005, mol/mol). It is noted here that, unless otherwise specified, SLBs used in these experiments were the first type. To prepare a lipid film, the solvent was vaporized with nitrogen gas flow at room temperature, and the film was put under vacuum for longer than 6 hr. To the vacuum-dried lipid film, 5 mL buffer L (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM DTT), that had been pre-warmed at 45℃, was added and stirred for 1 hr with a vortex mixer at 45℃ after nitrogen gas was injected and the tube was sealed. The solution was frozen using liquid nitrogen, and warmed again to 45℃. This freeze-thaw procedure was repeated five times. The resulting solution was passed 10 times through a polycarbonate filter (Cyclopore Polycarbonate Membranes, 0.1 μm pore size, Whatman, GE Healthcare, Little Chalfont, UK). It was then diluted with the same volume of buffer L and was sonicated at 28 kHz for 5 hr or more (5−8 hr). This long-time sonication is required to prevent the formation of a stable layer of absorbing vesicles that may be due to the failure to completely break the vesicles. Each vesicle suspension prepared was stored away from light at 4℃ after the nitrogen gas injection until use.

Planar lipid bilayer membranes were formed on a mica substrate as follows. The vesicle suspensions obtained as noted above were diluted with the same volume of buffer M10 (25 mM Tris-HCl, pH 8.0, 50 mM KCl, and 10 mM $CaCl_2$) and 400 μL of the diluted solution was dropped on the surface of the mica substrate (natural mica, Nilaco, Tokyo, Japan) that had been hydrophilized, and was incubated for 1 hr at 45℃. After 10 min incubation at room temperature, the substrates were washed 10 times with 400 μL buffer M5 (25 mM Tris-HCl, pH 8.0, 50 mM KCl, and 5 mM $CaCl_2$). Hereafter, all procedures were performed using ice-cold buffers. After

10 min incubation, each sample was washed 10 times with buffer M3 (25 mM Tris-HCl, pH 8.0, 50 mM KCl, and 3 mM $CaCl_2$), and was then immediately washed 5 times with buffer M1 (25 mM Tris-HCl, pH 8.0, 50 mM KCl, and 1 mM $CaCl_2$). After 10 min incubation, each sample was washed 10 times with buffer M (25 mM Tris-HCl, pH 8.0, 50 mM KCl), then washed 3 times with buffer ME (25 mM Tris-HCl, pH 8.0, 50 mM KCl, and 0.5 mM EDTA), and after 10 min incubation, washed 10 times with buffer M. In order to obtain SLBs without defects or multi-lamellar regions, a number of buffers were used serially for washing SLBs as described above. It is noted that mica instead of SiO_2/Si was used as the substrate for SLB in the above preparation method, in order to obtain SLBs that will not form defects even under Ca^{2+}-free conditions.

SLBs were observed using a fluorescence microscope (epi-fluorescence microscope BX51-FL, Olympus, Tokyo, Japan) with fluorescent lighting devices (X-Cite exact, EXFO, Quebec, Canada), a water immersion objective lens (LUMPlanFl, ×40, N.A. 0.80, Olympus) and a stage equipped with a temperature control. When BODIPY-PC was observed, a mirror unit (U-MWIG3, Olympus) was used. The excitation light intensity was adjusted by introducing 25 and 6% ND filters between the light source and the filter set. Images were obtained using a CCD camera (Coolsnaps ES, Photometrics, Tucson, AZ USA) and were stored using software (Meta Vue, Molecular Devices, Tokyo, Japan) as 16-bit grayscale TIFF files. The fluorescent images obtained as above were analyzed using Image J computer software (http://imagej.nih.gov/ij/). Prior to the experiments, SLBs obtained were confirmed for fluidity by Fluorescence Recovery After Photobleaching (FRAP) analysis.

Atomic force microscopy (AFM) of SLBs was performed with PicoScan2500 (Keysight Technologies, formerly Molecular Imaging, Tempe, AZ, USA) with a MAC lever Type VI cantilever. The probe used was 100 μm in length, 18 μm in width, 0.2 N/m of elastic constant

and 66 kHz of resonant frequency. AFM observation was carried out in a solution using Magnetic-AC mode, a kind of tapping mode. [37,38] The scanning speed was 0.1−1.0 line/sec, the resolution was 256 dots/line and the scan range was performed in 20 μm × 20 μm.

Temperature control was carried out using an aluminum handmade stage with a Peltier element (Netsu-denshi Kogyo, Tokyo, Japan) (Figure S1). The stage consists of two parts and holds the Peltier element between the bottom and upper parts. Water flows through the inside of the bottom part to cool the stage. Unless otherwise specified, the temperature of each SLB was changed stepwise by every 5℃, and the SLBs were kept at each temperature for 15 min. The reason to stand for 15 min is that, in the preliminary experiments, SLBs reached to a stable state within 10 min at the latest after changing the temperature.

The septin fraction obtained as described below was centrifuged (15,000 ×g for 20 min, 4℃) and was diluted two-fold with buffer M just prior to addition to the SLB, in order to remove the much larger septin polymers and/or the debris of denatured proteins. Four hundred μL of the septin fraction (protein concentration was 30 μg/mL) or the buffer solution resulting from the final dialysis outer solution (as a control) was applied, after its temperature was adjusted and buffer on the surface of each SLB was removed so as not to expose them to the air.

For each observation and numerical analysis, at least three independent experiments using different sets of SLBs and protein sample were performed.

2.3. Giant Liposomes

Giant liposomes were prepared by natural swelling as detailed previously. [30] In brief, 10 mM of each phospholipid stock solution in chloroform/methanol (98:2, v:v) were mixed (total 200 nmol of phospholipids). The lipid compositions of liposomes examined were PC alone (PC liposome),

PC:PI = 1:1 (PC+PI liposome), PC:PS = 1:1 (PC+PS liposome), PC:PG = 1:1 (PC+PG liposome) or DOPC:DMPS = 1:1 (mol:mol). The solvent was evaporated under a flow of nitrogen gas and the lipids were further dried in vacuo for at least 120 min. The dried lipid films were hydrated with 250 μL 150 mM sucrose at 50°C for 120 min, and the liposome suspensions were diluted 20-fold with buffer L.

Giant liposomes were observed by dark-field microscopy (BHF, Olympus) with an objective lens (HiApo40, Olympus) and a condenser (DC, Olympus). We manually tracked flowing liposomes in focus. The serial images were recorded using a DVD recorder (DMR-XW 120, Panasonic, Osaka, Japan). Fluorescent-labeled liposomes were observed using a fluorescence microscope (BH2-RFCA, Olympus) attached to the dark-field microscope.

2.4. Septin Fraction

The brain extract was prepared as reported previously. [30] In brief, the forebrain and cerebellum (about 90 gr) were dissected from a fresh porcine brain, washed with buffer A (25 mM Tris-HCl, pH 8.0, 2 mM EGTA, 500 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, and two protease inhibitor cocktail tablets), and then homogenized in 230 mL buffer A using a Waring blender. The homogenate was cleared by consecutive centrifugations at 5,000 ×g for 1 hr, 10,000 ×g for 1 hr and 171,000 ×g for 2 hr. The final supernatant was dialyzed against buffer L. The protein precipitate was salted out by adding ammonium sulfate to 40% (w/v) and incubation for 30 min, then collected by centrifugation at 10,000 ×g for 30 min and resuspended in buffer L (12 mL). The suspension was dialyzed against buffer L. After centrifugation at 171,000 ×g for 2 hr, the supernatant (termed the brain extract) was used either for further purification or was quickly

frozen with liquid nitrogen after addition of a final concentration of 1 mM GTP and stored at –80℃.

The brain extract was fractionated first with a cation exchange column HiTrap SP FF (GE Healthcare) attached to a FPLC liquid chromatography system (Pharmacia). The brain extract was charged on the column equilibrated with buffer C (25 mM Tris-HCl, pH 8.0, 10 mM NaCl), and bound proteins were eluted with a linear gradient of 10–500 mM NaCl in buffer C. The most active fraction was applied to a hydroxyapatite column, Econo-Pac CHT-II (Bio-Rad), and was eluted with a linear gradient of 10-500 mM potassium-phosphate buffer (pH 7.0). The major fractions were dialyzed against buffer L. After the dialysis, the fractions were measured for protein concentration using the BCA method with BSA as a standard, and were quickly frozen in the presence of a final concentration of 1 mM GTP with liquid nitrogen and stored at –80℃ until use. All procedures were conducted at 4℃ unless specifically noted.

In each step, fractions were applied to SDS-PAGE and assayed for liposome-transforming activity. A 10 μL liposome solution containing 40 μM phospholipid was placed in a glass flow cell at 25℃ and was observed using the dark-field microscope. Two μL of each sample solution, which had been dialyzed against buffer L, was placed into the flow cell by capillary force and the liposome suspensions were mixed by diffusion.

3. Results

3.1. Septin Arrays Assemble on the Surface of the Lipid Membrane

It is believed that septin is involved in the partition of biological membranes by restricting the lateral movements of lipids and proteins constituting the membranes. The partition of membranes is indispensable for the precise acceptance of complex signals by receptors and for

the exhibition of a variety of functions and the reinforcement of a unique morphology of individual cells.

It has been known that septin interacts with lipid membranes containing acidic phospholipids, and causes uniform bracing of the membrane as the result of the interaction. [30] In addition, we found in this study that septin can assemble into massive arrays of filaments on the surface of lipid membrane vesicles, regardless of the tubulation (Figure 1). The arrays of filaments formed on the membrane surface seemed to be involved in the compartment or domain formation of the membrane. Thus, in this study, we verified that hypothesis using SLBs.

3.2. Formation and Disappearance of Cooling-Induced Domains in SLBs in the Absence of Septin

Because SLBs are lipid membranes prepared on the surface of a flat substrate so that their fluidity is observable both by optical fluorescence microscopy and by AFM, it is a favorable experimental material to analyze the lateral movements of large numbers of molecules present in the membrane. [39, 40] In addition, it has been known that SLBs made from two synthetic phospholipids, DMPC and DOPC, form domains of the gel phase at temperatures lower than 12℃, because the Tc of DMPC and DOPC are different as mentioned above. [8]

In this study, we used SLBs that were made from PC and PI to investigate the effect of septin on lipid membranes other than the activity to induce membrane tubules, because PC and PI is the favorable composition for interactions between membranes and septin. [30] Incidentally, FRAP analysis showed that the SLBs prepared retain their fluidity at room temperature, either in the presence or absence of septin (Figure S2).

When the temperature was lower than 15℃, we found that SLBs form domains where the regions appear darker than the surrounding areas in fluorescent images (Figure 2 and Figure S3). In the process of decreasing the temperature, the domains that appeared around 15℃ increased in their size with further decreases of temperature. Since both the number and the size of domains did not change even when SLBs were placed at 5℃ for longer than 30 min, the domains formed were rather stable unless the temperature changed. Since most domains formed had a fractal shape, the domain formation might be from the diffusion-limited aggregation of lipid molecules.

Fluorescent-labeled annexin V is often used as a probe to detect PS on the outside of cell membranes, annexin V being a protein that can bind to acidic phospholipids, PI and PG as well as PS (Figure S4). If an SLB was made from PC and PI, the domains induced at a cold temperature, which are dark by fluorescent microscopy using a fluorescent-labeled lipid, were not recognized with the fluorescent annexin V (Figure S5 A–C). Conversely, the surrounding regions were recognized with that fluorescent annexin V. These observations suggest that the cooling-induced domains are PI-free regions. It should be noted here that the domain formation observed in this study is unique to the case of SLBs made from PC and PI obtained from natural sources. When the lipid composition of SLBs was PC and PS, which is another acidic phospholipid that is obtained from a natural source, the domain was not formed even if the SLB was kept at 5℃ (Figure S5D). Moreover, even though the head group of the phospholipids that constitute the SLBs is the same, the different configuration of the hydrocarbon chains can cause a different domain formation. When the lipid composition of the SLBs was DOPC and DMPS, the regions observed as dark by fluorescent microscopy using a fluorescent-labeled lipid appeared in the cooled SLBs, while the regions were recognized with fluorescent-labeled annexin V (Figure S5E). Since the conditions and mechanism for the formation of the cooling-

induced domain are complicated, the domains were used simply as a tool to examine the effect of septin on the dynamics of lipid membranes in this study.

The cooling-induced domains disappeared reversibly by increasing the temperature (Figures 2 and S3). When the temperature was increased from 5 to 10℃, another region that possesses a bright intensity in fluorescent images, penetrated into the domains from the surrounding areas, resulting in the dispersion of the domains (Figure 2 and Figure S3, and see also Figure 4). Thus, the number, and also the total area in some cases, of the domains increased once in the process of increasing the temperature, even though the domain size, that is the average size of the individual domains, decreased. When the temperature was higher than 15℃, the total area of domains decreased with increasing temperature (Figure 3). When the temperature reached 25℃, the domains completely disappeared.

3.3. Effect of Septin on the Formation of the Domains

When septin was added to an SLB at 25℃, *i.e.*, the solution covering an SLB was exchanged with a solution containing septin prior to starting the process of decreasing the temperature of the SLB, the size of the cooling-induced domains formed in the SLB was extremely restricted to a much smaller size and inversely their number was increased, which resulted in a decrease of the total area of the domains formed (Figures 2 and 3). When the solution on an SLB was exchanged with a solution under the same conditions but without septin, these differences were never induced. Therefore, the differences in results between cases in the presence or absence of septin result from the effect of septin on the formation of the cooling-induced domain in SLBs.

The individual domains retained a fractal shape, regardless of the presence of septin (Figure 2). The temperature when the domains appeared in the process of decreasing the temperature (about

15℃) and the temperature when the domains completely disappeared in the process of increasing the temperature (20−25℃) was not affected by septin (Figure 3). Altogether, septin affects the size and number, but not the basis of the formation, of the cooling-induced domain. Septin may inhibit the movement of PC molecules toward the domains that had already appeared in the SLBs and/or PI molecules from the surroundings of the domains via binding with PI molecules.

3.4. Effect of Septin on the Disappearance of the Domains

When septin was added to an SLB at 5℃, *i.e.*, the solution covering an SLB was exchanged with a solution containing septin after the formation of cooling-induced domains and before starting the process of increasing the temperature, the cooling-induced domains that formed in the SLB became very stable against the temperature increase and tended to remain even after the SLB was placed at 25℃ for over than 60 min (Figures 4 and 5). Also, in this case, exchanging the solution on the SLB with a solution with the same conditions but without septin, never induced the difference. Therefore, the difference in results between cases in the presence or absence of septin results from the effect of septin on the disappearance of the cooling-induced domain that formed in SLBs.

Septin robustly prevented the shrinking and dispersion of the domains (Figure 4). Thus, during the temperature increasing process, the shape of the individual domains was maintained (Figure 4) and changes in all parameters, *i.e.* the domain size, the number of domains, and the total area of the domains, were extremely delayed by the addition of septin (Figure 5). Septin may inhibit the movement of lipid molecules constituting the SLB via binding with PI molecules.

3.5. Septin on the Surface of SLBs

Because septin is thought to inhibit the movement of lipid molecules within an SLB as described above, to determine the mechanism involved, next we tried to observe septin interacting with SLBs using AFM (Figure 6). The SLBs prepared usually had a flat surface (Figure 6A and B), except rarely showed some very small protrusions (Figure 6C) or defects (Figure 6D).

When septin was added, although the fluorescent images did not show remarkable difference (Figure 6E), some objects tended to be observed on the surface of SLBs by AFM (Figure 6F and G). They were string-like structures or round-shape protrusions, which have a width or length ranging from tens of nanometers to several micrometers and a height of about 5 nm. Septin forms a hetero-hexamer, which consists of two hetero-trimers arranged in tandem, as a unit complex for polymerization. The septin complex then polymerizes to filaments with a diameter of about 5 nm, and subsequently those filaments associate with each other in a side-by-side manner to form larger arrays. Therefore, the objects found on the surface of SLBs are assumed to be assemblies of septin complexes. Membrane tubulation or the formation of long septin filaments was not observed, which is reason that the amounts of septin added were kept low in order to protect SLBs from decay caused by their deformation. The size of their lateral directions and the shape of the assembly were not dependent on the amount of septin applied (Figure 6G). Depending on the location on the SLBs rather than the amount of septin, various structures, *i.e.*, string-like structures or round-shape protrusions, are present. The reason may be due to the formation of varied assemblies in each experiment because the nucleation that is a critical step for the polymerization is a labile process. In addition, in the concentration ranges of septin examined here, no remarkable difference was found in the septin-applied SLBs and the same interference effects of septin on the SLBs were observed, even when the concentrations of septin

added were changed. With increasing of the septin concentration, only the reproducibility of the results was increased.

4. Discussion

4.1. Cooling-Induced Domains Formed in SLBs

Because both the BODIPY-PC and TR-DHPE used preferentially partition into a liquid crystal phase in SLBs that causes phase separation, the bright region in fluorescent images that appears at low temperatures is thought to be in the liquid crystal phase. [36] The remaining regions, which are dark in fluorescent images and were confirmed not to be defects by AFM, that is, the cooling-induced domains may be in the gel phase. In addition, as mentioned above, the observations using fluorescent-labeled annexin V suggest that the domains are PI-free regions.

The domain formation observed in the cooled SLBs depends on the charge or size of the head group and the length or degree of unsaturation of the hydrocarbon chains of the phospholipids contained in the membrane and on the diversity (or uniformity) of the phospholipids constituting the membrane, and is caused by a complicated involvement of these factors. The shape of the domain suggests that the domain formation may result from the diffusion-limited aggregation of phospholipids. [41] Since the conditions and mechanism for the formation of the cooling-induced domain found in this study remain unclear, they should be further investigated. Moreover, the lipid composition of the SLBs used in this study and the condition for the domain formation are different from the naturally occurring. Therefore, studies using membrane domains that can be induced under more physiological conditions should be required.

4.2. Effect of Septin on the Formation and Disappearance of the Domain

Septin may inhibit the diffusion-propelled long-range movements of lipids via the formation of some complexes with PI molecules on the SLBs (Figure 7). If septin is added to an SLB before the temperature decrease, *i.e.*, prior to the domain formation, the inhibitory effect of septin would stagnate the movement of PI molecules receding from the domain and thus restrict the number of PC molecules gathering per domain. As a result, in comparison with the case without the addition of septin, the domains that appear during the process of the temperature decrease remain small without growth and increase their number without coalescence (Figure 2). In contrast, if septin is added to an SLB after the domain formation caused by the temperature decrease, the inhibitory effect of septin would stagnate the movement of PI molecules penetrating to the domain region and thus obstruct the movement of PC molecules leaving the domain. As a result, in comparison with the case without the addition of septin, the domains show a delayed disappearance during the process of the temperature increase (Figure 4).

As described above, the domain is estimated as a PI-free region. Septin interacts with PI but not with PC. Together, these results suggest that septin interacts with the domain-forming SLB and is distributed outside the domain region. Therefore, the complexes consisting of septin proteins on the surface of the SLBs and PI lipids in the SLBs, are thought to be formed outside the domain regions (Figure 7). On the surface of the SLBs, septin proteins polymerize to form filaments, or assemble to form oligomers, for example, nuclei that are the previous stage of filament elongation. Against the diffusion movements of phospholipids in the SLBs, the complexes can play the role of a fence in the former case (the upper model in Figure 7) and the pickets in the latter case (the bottom model in Figure 7). Unfortunately, the AFM images obtained show that there are both fibrous structures and round-shape protrusions on the septin-treated SLBs, suggesting the possibility that both structures occur, and thus we could not reach a

decision about this (Figure 6). In either case, electrostatic interactions between septin and acidic lipids may be important for the formation of the diffusion barrier in the lipid membrane, as reported previously by a simulation study. [33] That study also indicated that adequate blockage against diffusion by a protein complex placed on the membrane surface is hard to provide, which is consistent with the results of the FRAP analysis of SLBs at room temperature, which were not affected by septin (Figure S2). Possibly, septins interacting with PI lipids can block the movement of the populations of lipids, which are clustered by the interaction, but not the diffusion of individual lipid molecules.

The domain formation investigated here is particular to the conditions of low temperature. The mechanism whereby septin affects the formation and disappearance of the cooling-induced domain remains unknown, whether the fence by filaments or the pickets by oligomers. In any case, the results obtained in this study clearly demonstrate that septin, a membrane-interacting cytoskeletal protein, can affect the size, number, and stability of the cooling-induced domain that is induced in the membrane.

Septin has been estimated to not only be involved in the reinforcement of membrane structures, but also to play a role as a diffusion barrier against membrane proteins in diverse types of cells such as at the neck of sperm or the base of spinal nerve synapses. [42,43] The effects of septin on the membrane domain as directly observed in our *in vitro* study here strengthens the earlier suggestion of the involvement of septin in the partitioning of cell membranes proposed by *in vivo* and *in silico* studies. The ability of septin as demonstrated here will enable us to regulate the domain formation of lipid bilayer membranes, although the detailed mechanism involved remains unclear. In addition to septin, many other proteins have been shown to interact with

membranes and to assemble at their surface. Those proteins might also be useful for the control of the properties of lipid bilayer membranes.

5. Conclusions

Free control of processes related to the partitioning of lipid membranes, including phase separation and domain formation, is very important physiologically as well as physically and is an engineering issue that allows the diversification of membrane function to evolve. In this study, we investigated the effect of septin on lipid bilayer membranes using SLBs, and found that PI-containing SLBs reversibly form domains when the temperature is decreased, and that septin affects both the formation and the disappearance of the cooling-induced domain. Septin inhibits the growth of those domains during the decrease of temperature, and inhibits the dispersion and disappearance of the domains during the increases of temperature. The effects of septin on the membrane domain directly observed in this study strengthen the hypothesis of the physiological role of septin concerning the partitioning of cell membranes. These functions of septin should enable the regulation of domain formation and the creation of unique regions in lipid bilayer membranes.

FIGURE CAPTIONS

Figure 1. Giant liposome coiled with septin filaments. Electron micrographs show a PC+PI liposome in the absence (left) or presence (center) of the septin fraction. Arrows indicate regions that are expected to begin to tubulation. Septin filaments are wrapped around these short tubules. The right panel shows an enlarged and contrast enhanced image of the region indicated by the blue box in the center panel, in order to show bundles of septin filaments assembled on the

surface of the liposome. Some of the bundles are indicated by blue brackets. Bars indicate 200 nm.

Figure 2. Effect of septin on the cooling-induced domain formation in SLBs made from PC, PI, and fluorescence-labeled PC. A buffer solution without (upper) or with septin (bottom) that had been adjusted to 25℃ was applied to an SLB. The temperature was then decreased from 25 to 5℃ by 5℃ every 15 min. After standing at 5℃ for 30 min, the temperature was increased to 25℃ by 5℃ every 15 min. Fluorescent images of SLBs at 25 and 15℃ during the temperature decreasing process and at 5, 15, and 25℃ during the temperature increasing process are shown. Bars indicate 10 μm. The same field of SLBs was continuously observed. More detail is shown in Figure S3 regarding the case without septin (controls).

Figure 3. Size (top), number (middle), and total area (bottom) of the cooling-induced domains formed in SLBs in the presence (red: '+ Septin') or the absence of septin (blue: 'Control'). Conditions are the same as detailed for Figures 2 and S3; that is, a buffer solution without or with septin, that had been adjusted to 25℃, was added to the SLBs before changing the temperature. The size (μm^2) of the domains formed is shown by boxplot. The top and bottom of each whisker indicate the maximum and minimum values, the upper and lower ends of each box indicate the first and third quarter points, and the white line in the box represents the median. The number of domains formed and the ratio (%) of total area of the domains against the total area of SLBs are shown by line graphs. Error bars indicate standard deviation (S.D.). When

results obtained in the presence of septin are compared with those in the absence of septin, the p values of <0.0001, 0.001, and 0.01 are indicated by ***, **, and *, respectively. The Wilcoxon-Mann-Whitney signed-rank test was used for statistical analysis.

Figure 4. Effect of septin on the cooling-induced domain formed in SLBs. SLBs were made from PC, PI, and fluorescence-labeled PC. The temperature of the SLBs was decreased to 5℃ and the cooling-induced domain was formed. A buffer solution without (upper) or with septin (bottom) that was at 0℃ was then applied to the SLBs. After standing at 5℃ for 90 min, the temperature was increased to 25℃ by 5℃ every 15 min, then left for up to 120 min at 25℃. Fluorescent images of SLBs at 5, 10, 15, and 25℃ during the temperature increase are shown in the case of the absence of septin, and images at 5, 10, and 15℃ during the temperature increase and after standing for 60 min at 25℃ are shown in the case of the presence of septin. Bars indicate 10 μm.

Figure 5. Size (top), number (middle), and total area (bottom) of the cooling-induced domains formed in SLBs in the presence (red: '+ Septin') or absence of septin (blue: 'Control'). Conditions are the same as detailed for Figure 4; that is, after SLBs were cooled down to 5℃ and the cooling-induced domain was formed, a buffer solution without or with septin that was at 0℃, was added to the SLBs. The size (μm^2) of domains formed is shown by boxplot. The number of domains formed and the ratio (%) of total area of the domains against the total area of SLBs are shown by line graphs. In the middle and bottom panels, when results obtained in the presence of

septin are compared with those in the absence of septin, the p values of <0.0001, 0.001, and 0.01 are indicated by ***, **, and *, respectively.

Figure 6. Properties of the SLB surface analyzed by fluorescence microscopy and by AFM. SLBs were made from PC, PI, and fluorescence-labeled PC. SLBs without (A−D) or with septin (E−G) were observed by fluorescence microscopy (A and E) or by AFM (B−D, F, and G). The bar in each fluorescent image indicates 10 μm. The vertical and horizontal range shown in the topographic image of AFM is 2 μm × 2 μm (B−D) or 20 μm × 20 μm (F and G). The color scale on the right side of each topographic image indicates the height of the SLB surface (nm). (B) Results of an SLB, which has an almost flat surface. (C) Results for the case when some particles were found on the SLB surface. (D) Results of an SLB, which has defects. The final amounts of septin added were 6.8 (E), 12 (F), 0.85, 6.8, 10, and 12 μg (from left to right in G). In the case of the AFM observation of SLB shown here, a septin solution stored at 0℃ was added to an SLB incubated at 25℃. Observations were performed at 25℃. In (C), (D), and (F), the height of the SLB surface (nm) obtained by the line scanning in the location indicated by the white line in the topographic image is also shown.

Figure 7. Model for the effect of septin on the formation and disappearance of the cooling-induced domain in SLBs; i) septin filament behaving as a fence suppressing the lateral diffusion of phospholipids (upper two panels), and ii) septin facilitating the nucleation by binding to the membrane and tethering PI molecules (lower two panels). In each model, the upper layer is the

case when septin is added to an SLB incubated at 25℃ and thus has not yet formed the domain, and the bottom layer is the case when septin is added to an SLB incubated at 5℃ and thus has already formed the domain. Phospholipids shown in orange and red are PI and PC, respectively. We estimate that the cooling-induced domain, which has a lower fluorescent intensity, is a PI-free region. Blue indicates septin filaments or oligomers (nucleus) that are assembling on the membrane. Septin can interact with the lipid bilayer membrane via associating with PI, an acidic phospholipid. The concentrations of septin added were restricted so as not to induce membrane tubulation because membrane tubulation causes defects in SLBs. In such concentration ranges, septin might not assemble into filaments. Septin did not alter the result of FRAP analysis that was carried out at room temperature (Figure S2). Therefore, septin proteins might be in a state that allows diffusion together with lipid molecules. Altogether, the model shown in the lower half might be more accurate than that shown in the upper half.

ASSOCIATED CONTENT

Supporting Information

Photograph of the stage of the fluorescence microscope. Sequences of fluorescent images showing typical FRAP results of SLBs prepared. Sequence of fluorescent images showing the cooling-induced domain formed in SLBs made from PC, PI, and fluorescence-labeled PC. Dark-field and fluorescent images of giant liposomes in the presence of fluorescent-labeled annexin V. The distribution of the cooling-induced domain and the distribution of SLB-binding annexin V. This information is available free of charge via the Internet at http://pubs.acs.org.

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Conflict of Interest

The authors declare no competing financial interest.

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ABBREVIATIONS

SLB, supported lipid bilayer; AFM, atomic force microscopy; PI, phosphatidylinositol; TR-DHPE, N-(Texas Red sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; BODIPY-PC, 2-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-

hexadecanoyl-sn-glycero-3-phosphocholine; PC, phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylserine.

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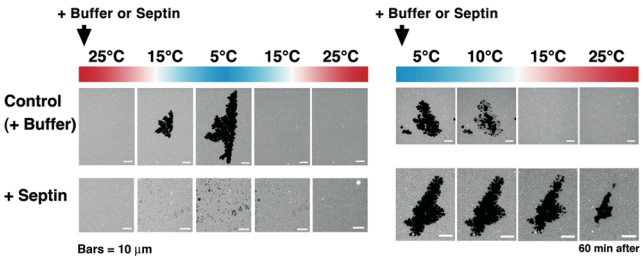

Septin is one of the proteins that form a cytoskeletal network in biological membranes. A membrane domain formation is inducible in supported lipid bilayers (SLBs) consisting of phosphatidylcholine and phosphatidylinositol by cooling. In the presence of septin, the formation and disappearance of the cooling-induced domain is affected. This finding demonstrates that it is possible to regulate membrane domain formation by utilizing the membrane beneath the cytoskeletal protein.

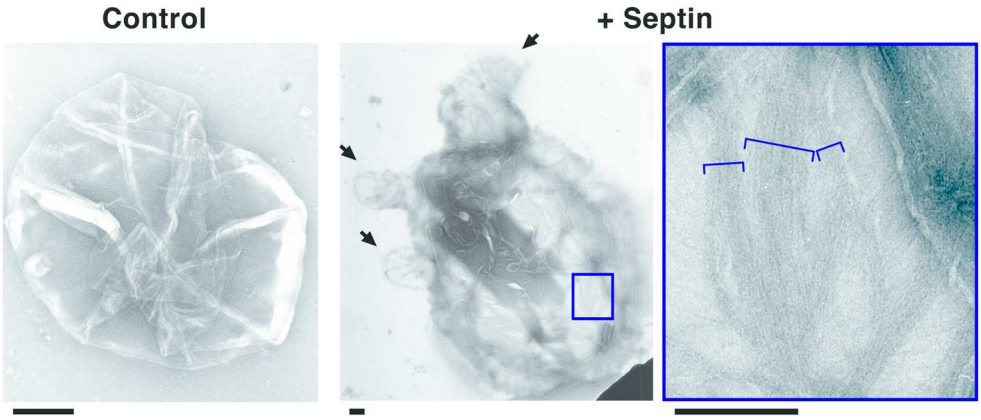

Figure 1. Giant liposome coiled with septin filaments. Electron micrographs show a PC+PI liposome in the absence (left) or presence (center) of the septin fraction. Arrows indicate regions that are expected to begin to tubulation. Septin filaments are wrapped around these short tubules. The right panel shows an enlarged and contrast enhanced image of the region indicated by the blue box in the center panel, in order to show bundles of septin filaments assembled on the surface of the liposome. Some of the bundles are indicated by blue brackets. Bars indicate 200 nm.

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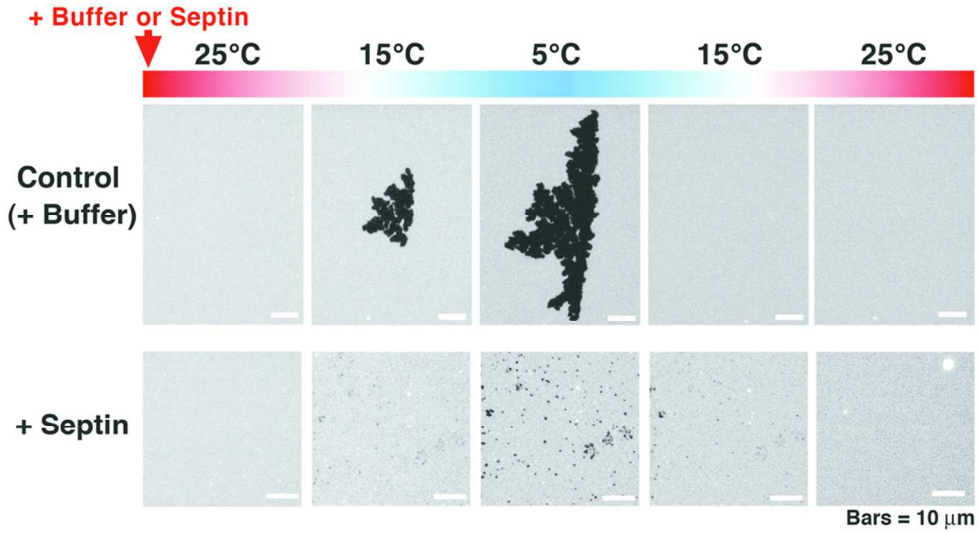

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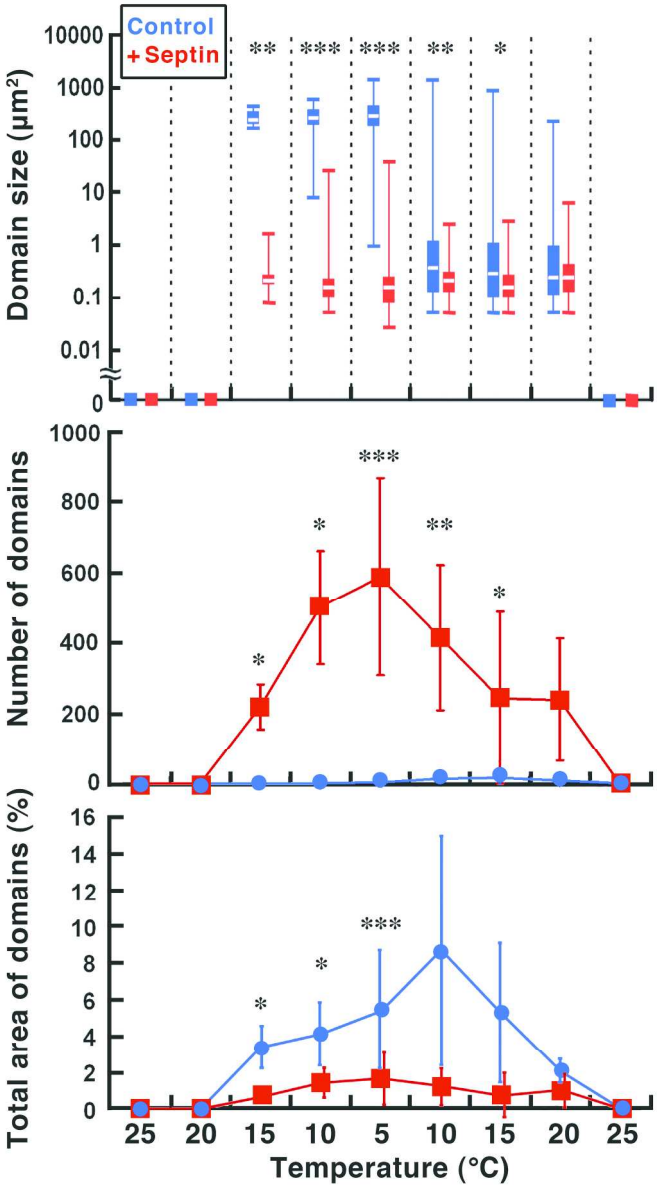

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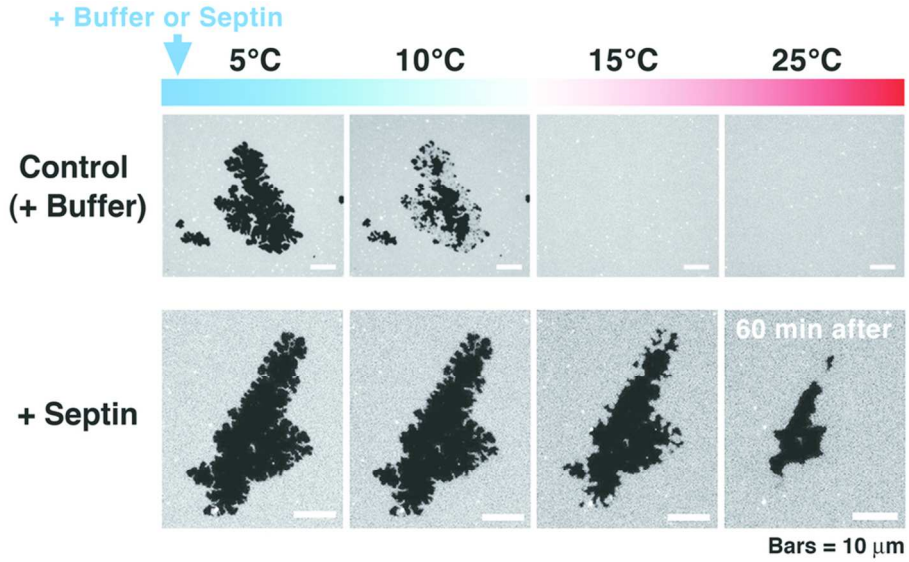
149x264mm (300 x 300 DPI)



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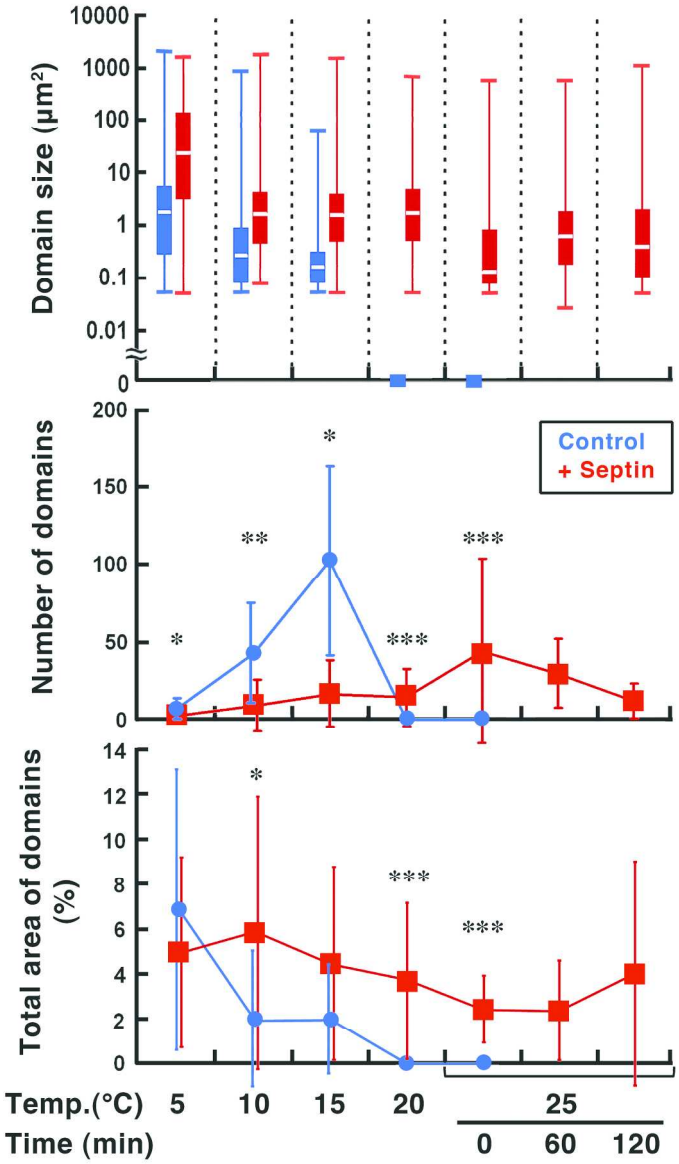
90x50mm (300 x 300 DPI)



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150x264mm (300 x 300 DPI)

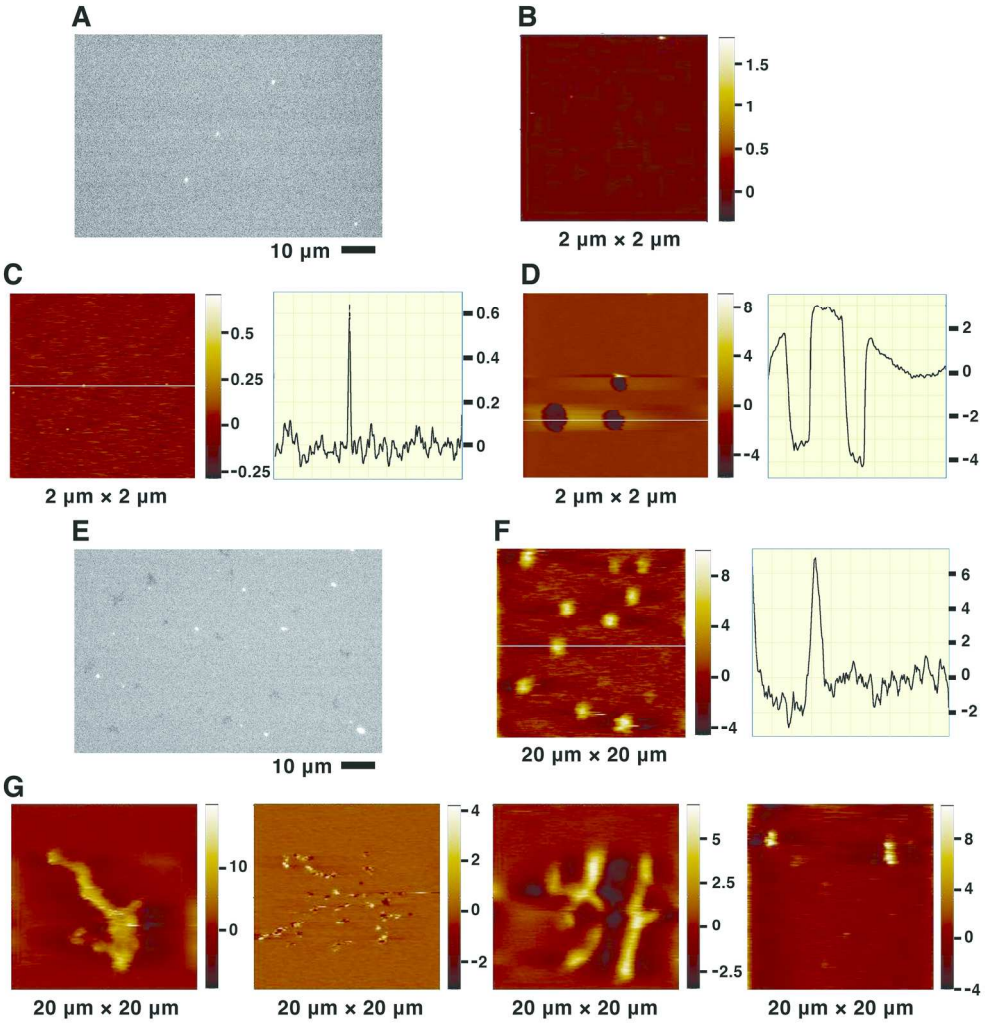

Figure 6. Properties of the SLB surface analyzed by fluorescence microscopy and by AFM. SLBs were made from PC, PI, and fluorescence-labeled PC. SLBs without (A−D) or with septin (E−G) were observed by fluorescence microscopy (A and E) or by AFM (B−D, F, and G). The bar in each fluorescent image indicates 10 μm. The vertical and horizontal range shown in the topographic image of AFM is 2 μm × 2 μm (B−D) or 20 μm × 20 μm (F and G). The color scale on the right side of each topographic image indicates the height of the SLB surface (nm). (B) Results of an SLB, which has an almost flat surface. (C) Results for the case when some particles were found on the SLB surface. (D) Results of an SLB, which has defects. The final amounts of septin added were 6.8 (E), 12 (F), 0.85, 6.8, 10, and 12 μg (from left to right in G). In the case of the AFM observation of SLB shown here, a septin solution stored at 0℃ was added to an SLB incubated at 25℃. Observations were performed at 25℃. In (C), (D), and (F), the height of the SLB surface (nm) obtained by the line scanning in the location indicated by the white line in the topographic image is also shown.

180x185mm (300 x 300 DPI)

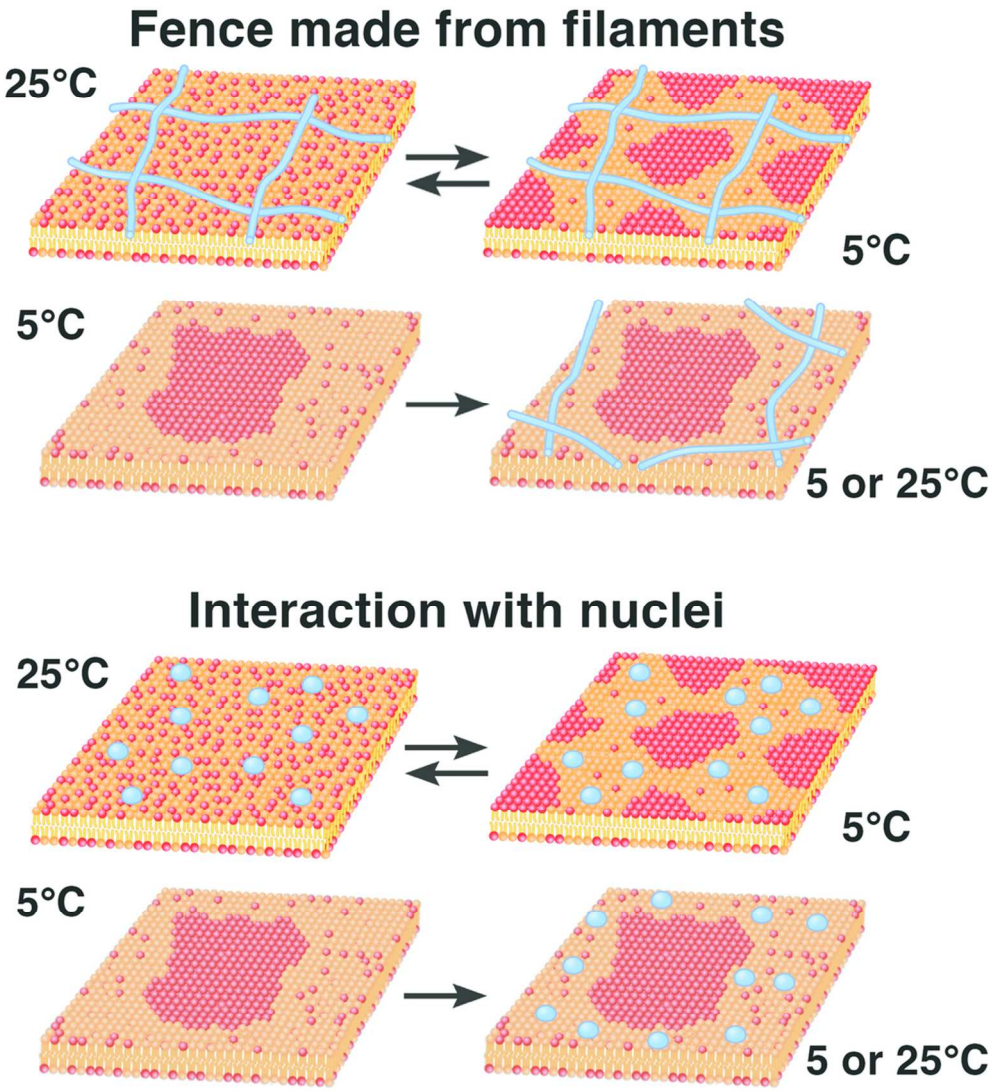

Figure 7. Model for the effect of septin on the formation and disappearance of the cooling-induced domain in SLBs; i) septin filament behaving as a fence suppressing the lateral diffusion of phospholipids (upper two panels), and ii) septin facilitating the nucleation by binding to the membrane and tethering PI molecules (lower two panels). In each model, the upper layer is the case when septin is added to an SLB incubated at 25℃ and thus has not yet formed the domain, and the bottom layer is the case when septin is added to an SLB incubated at 5℃ and thus has already formed the domain. Phospholipids shown in orange and red are PI and PC, respectively. We estimate that the cooling-induced domain, which has a lower fluorescent intensity, is a PI-free region. Blue indicates septin filaments or oligomers (nucleus) that are assembling on the membrane. Septin can interact with the lipid bilayer membrane via associating with PI, an acidic phospholipid. The concentrations of septin added were restricted so as not to induce membrane tubulation because membrane tubulation causes defects in SLBs. In such concentration ranges, septin might not assemble into filaments. Septin did not alter the result of FRAP analysis that was carried out at room temperature (Figure S2). Therefore, septin proteins might be in a state that allows diffusion together with lipid molecules. Altogether, the model shown in the lower half might be more accurate than that shown in the upper half.

94x106mm (300 x 300 DPI)