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Cholesterol-Dependent Phase-Demixing in Lipid Bilayers as a Switch for the Activity of the Phosphoinositide-Binding Cytoskeletal Protein Gelsolin

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Abstract

The lateral distribution of phosphatidylinositol 4,5-bisphosphate (PIP₂) in lipid bilayers is affected both by divalent cation-mediated attractions and cholesterol-dependent phase demixing. The effects of lateral redistribution of PIP₂ within a membrane on PIP₂-protein interactions are explored with an N-terminal fragment of gelsolin (NtGSN) that severs actin in a Ca²⁺-insensitive manner. The extent of NtGSN inhibition by PIP₂-containing large unilamellar vesicles (LUVs) depends on the lateral organization of the membrane as quantified by an actin-severing assay. At a fixed PIP₂ mole fraction, the inhibition is largely enhanced by the segregation of liquid ordered/liquid disordered (L_o/L_d) phases that is induced by altering either cholesterol content or temperature, whereas the presence of Ca²⁺ only slightly improves the inhibition. Inhibition of gelsolin induced by demixed LUVs is more effective with decreasing temperature, coincident with increasing membrane order as determined by Laurdan generalized polarization and is reversible as the temperature increases. This result suggests that PIP₂-mediated inhibition of gelsolin function depends not only on changes in global concentration but also on lateral distribution of PIP₂. These observations imply that gelsolin, and perhaps other PIP₂-regulated proteins, can be activated or inactivated by the formation of nanodomains or clusters without changing PIP₂ bulk concentration in the cell membrane.

Graphical Abstract

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Phosphatidylinositol 4,5-bisphosphate (PIP₂) is involved in actin cytoskeletal remodeling in many ways. Such remodeling is mediated by PIP₂ through its direct interaction with actin-binding proteins^{1–3} or indirectly by its effects on small GTPases and other factors that regulate actin assembly.⁴ Cortical actin reorganization is a dynamic process regulated by PIP₂ on both global and local levels.^{5,6} The mechanism by which PIP₂ locally regulates actin assembly remains unclear. Recent studies have revealed an inhomogeneous lateral distribution of PIP₂ in lipid bilayers,^{7–9} and advances in optical microscopy further reveal the formation of PIP₂ nanoclusters in cell plasma membranes^{10,11} with dimensions similar to those imaged by atomic force microscopy in purified PIP₂-containing membranes.¹² While many hypotheses have been proposed to explain the physical chemical principles behind the formation of PIP₂ lateral inhomogeneity either in plasma or model membranes, whether PIP₂-mediated cellular functions can be regulated by local perturbations of PIP₂ lateral distribution is still an open question.

Among the first reported PIP₂-associated actin-regulating proteins, gelsolin is a well-characterized autoinhibited protein that is activated at low pH or by Ca²⁺ and is inhibited by PIP₂. Gelsolin affects actin reorganization by severing actin filaments,¹³ capping the fast growing ends of actin filaments¹⁴ and producing nucleation sites for new actin filament formation.¹⁵ The detailed biochemical functions of gelsolin are reviewed elsewhere.^{16–21} PIP₂-inhibited activity of gelsolin has been extensively investigated using pyrene-labeled actin assembly and depolymerization assays.²² The actin-severing activity of gelsolin is strongly affected by PIP₂ in a micellar form, for which half maximal inhibition is achieved at 1.7 μ M PIP₂.²³ Early studies suggest that the sensitivity of gelsolin to PIP₂ is subject to the physical states of PIP₂ in a membrane.²³ PIP₂-mediated gelsolin inhibition requires greater total PIP₂ concentrations when it is mixed with so-called “vesicle-forming” lipids, which presumably induce a different lipid packing geometry for PIP₂ compared to its conformation in micelles. A reduced PIP₂ inhibiting capability in the presence of other lipids is restored through extensive sonication²⁴ after which PIP₂ presents most likely in the form of small unilamellar vesicles.²⁵ The interactions between gelsolin and PIP₂ in bilayer membranes with different lipid lateral organizations such as those caused by changes in cholesterol content have not yet been determined. Effects of PIP₂ lateral distribution on gelsolin’s ability to bind actin would have potential relevance to the mechanisms by which the severing and capping of actin is locally controlled in cells.

In this study, we investigate PIP₂–gelsolin interactions in cholesterol-dependent phase-demixed large unilamellar vesicles in an actin-severing assay. The perturbation in PIP₂ lateral organization is achieved either by adding divalent cations or changing the

temperature. Because full-length gelsolin is sensitive to the presence of Ca^{2+} , a Ca^{2+} -insensitive N-terminal half of the gelsolin (NtGSN)^{13,26} was used so that divalent cation-induced effects on the lipids can be studied without confounding effects on the protein. Similar N-terminal gelsolin fragments are generated by caspase 3 and remain sensitive to PIP_2 .²⁶ The findings from our study could improve our understanding of the links between PIP_2 signaling and dynamic local response at the cell membrane/cytoskeletal interface.

EXPERIMENTAL PROCEDURES

Lipids and Reagents

Dioleoyl- $\text{PI}(4,5)\text{P}_2$ and neutral phospholipids such as dioleoylphosphatidylcholine (DOPC), dipalmitoyl-phosphatidylcholine (DPPC), and Topfluor Cholesterol (23-(dipyrrometheneboron difluoride)-24-norcholesterol were purchased from Avanti (Alabaster, AL). GloPIPs BODIPY TMR- $\text{PI}(4,5)\text{P}_2$ C16 was purchased from Echelon Biosciences (Salt Lake City, UT). The concentrations of unlabeled lipid stock solutions were routinely monitored by a phosphorus assay as described elsewhere.²⁷ Tris, EGTA, CaCl_2 , and KCl were purchased from Fisher Scientific (Hampton, NH). Laurdan was from AnaSpec Inc. (Fremont, CA), and dithiothreitol (DTT) was purchased from Research Product Int. Corp. (Mt. Prospect, IL). Dihydrocholesterol (DChol) was purchased from Sigma-Aldrich (St. Louis, MO) and was used instead of cholesterol to avoid unwanted artifacts resulting from the photo-oxidation of cholesterol.^{28–30}

Protein Purification

Actin from rabbit muscle³¹ and full-length gelsolin from human blood plasma³² were purified according to published methods, and actin was labeled with *N*-(1-pyrenyl)iodoacetamide as previously described²² with 93% labeling efficiency. 6.2 mg/mL Pyr-G-actin in solutions containing 2 mM Tris, pH 8.0, 0.2 mM CaCl_2 , 0.5 mM dithiothreitol, 0.5 mM ATP (G-buffer) were frozen in aliquots. Pyrene G-actin was thawed before use and followed by a 15-fold dilution in a G-buffer for at least 30 min to depolymerize the actin. Actin was then polymerized at room temperature by supplementing the buffer with 2 mM MgCl_2 and 150 mM KCl to form F-buffer. The prepared F-actin sample was used within 2–3 days.

The amino-terminal gelsolin (NtGSN) was purified from *Escherichia coli*. Mouse gelsolin (UniProt# Q6PAC1) fragment from 2 to 351 amino acids was encoded in a pQE30 vector with a His-tag attached to the N-terminal of the protein. The construct was transformed into and expressed by XL1-blue, instead of BL21 DE3 competent cells, as the proliferation of similarly transformed BL21 DE3 cells was greatly suppressed. NtGSN was purified using His-Bind Quick 900 cartridges (Novagen, Madison, WI). The elution buffer was replaced by an NtGSN buffer, which contains 10 mM Tris, 0.4 mM CaCl_2 , and 150 mM NaCl at pH7.0, using a HiTrap desalting column (GE Healthcare) noticing NtGSN precipitates within hours in the absence of Ca^{2+} at 4 °C.³³ The final product has 357 amino acids with a 40 kDa molecular mass. The extinction coefficient of NtGSN at 280 nm is $47\,500\text{ cm}^{-1}\text{ M}^{-1}$ based on its sequence.

LUV Preparation

Regardless of PIP₂ mole fraction, the total amount of PIP₂ used in LUV preparation was set as a constant for all PIP₂-containing LUVs. Therefore, depending on the PIP₂ mole fraction, a total of 0.24 or 0.72 μmol of lipid mixture at a desired composition was mixed in chloroform in a glass test tube and blow-dried under nitrogen. Traces of organic solvent were removed by vacuum drying for at least 2 h. Subsequently, the dried lipid film was rehydrated with 100 μL of buffer containing 2 mM Tris, 0.5 mM dithiothreitol, 150 mM KCl at pH7.0. The rehydrated lipid film was then sonicated in a water bath sonicator (Fisher Scientific, Pittsburgh, PA) for 10 min, and extruded through a polycarbonate membrane with an average pore size of 200 nm (Avestin, Ottawa, CA) using a mini-extruder (Avanti, Alabaster, AL) on a hot plate at 60 °C. The effective PIP₂ concentration for all LUVs stock solutions was 180 μM which takes into account only the PIP₂ in the outer leaflet. Because of the small volume used in mini-extruder, the lipid resuspension was extruded 51 times to ensure proper mixing.

PIP₂-containing LUVs in fully mixed bilayers (LUV B) or bilayers that demix to different extents (LUV A, C, and D) were prepared based on a known phase diagram for a ternary lipid mixture containing DOPC/DPPC/DChol.³⁴ Because PIP₂ has been reported to partition preferentially into the liquid disordered phase,³⁵ the mole fraction of PIP₂ is added to that of DOPC in this phase diagram. The lipid compositions (PIP₂/DOPC/DChol/DPPC) of four different LUVs used in this study are LUV A = 15/10/30/45; LUV B = 15/80/0/0; LUV C = 5/20/30/45; LUV D = 5/10/30/55. (See also Figure 1E.)

Gelsolin Severing Assay

This assay follows the method reported earlier¹⁵ with some modifications. A final concentration of 31 nM NtGSN was incubated with PIP₂-containing LUVs at a desired concentration in an F-buffer for 4 min at a desired temperature before pyrene-F-actin was added. A final concentration of 0.37 μM pyrene-F-actin was added into the premix and rapidly vortexed before the sample was transferred to a recording fluorescence reader. The collection of emission time courses started before pyrene actin was added so the time point at which pyrene actin is added (t_0) is well controlled ($t_0 = 4$ s) and the delay between mixing and the initial fluorescence signal varies between 6 and 8 s. The decay in pyrene fluorescence was measured using an LS-50B fluorescence spectrometer coupled with a four-position water thermostated cell holder (PerkinElmer, Waltham, MA). The temperature is controlled by a 9110-VT1 recirculator water bath (PolyScience, Warrington, PA).

The sample volume was fixed at 50 μL so the measurements at a higher PIP₂ concentration up to 108 μM could be achieved at acceptable cost. The volumes of NtGSN and pyrene-F-actin added were less than 5% of the total volume so the Ca²⁺ carried from both protein solutions was negligible. For some experimental conditions, F-buffer that contains 0.2 mM Ca²⁺ was replaced with buffer supplemented with 1 mM EGTA (FE buffer) to investigate effects of Ca²⁺-induced perturbation in PIP₂ lateral distributions. The difference in the nominal temperature in the water bath and the terminal temperature inside the cell holder was calibrated using a thermocouple (Barnant, Barrington, IL). The lower limit of the terminal temperature was set at 11 °C because condensation of water on sample glass tubes

interfered with the measurement at a low temperature, and the upper limit is limited by the recirculator. The outer surface of the cell holder was taped with an open slit so that only the middle section of the 50 μ L mixed solution was exposed and excited.

Fluorescence Time Course Analysis

Changes in pyrene fluorescence were fitted into a single exponential decay:

$$F = F_0 - (F_0 - F_b) \times [1 - e^{-k_1(t-t_0)}] \quad (1)$$

The representative fittings of the actin depolymerization kinetics are shown as yellow lines in Figure 1A. The level of gelsolin inhibition was quantified by the initial disassembly rate of pyrene-F-actin, which is proportional to the number of free pointed ends of actin filaments, most of which were generated by the concentration-dependent severing of actin filaments by gelsolin.¹⁵ The initial fluorescence decay rate at $t = t_0$ was calculated as $k_1(F_0 - F_b)$ based on the fitting results. Noticeably, the final fluorescence F_b increases as LUV concentration increases, so the data were normalized by subtracting the background fluorescence to a same F_0 , as shown in Figure 1A–D.

Steady-State Probe-Partitioning FRET on Large Unilamellar Vesicles (LUVs)

The phase partitioning of PIP₂ and DChol in a phase-demixed membrane at various ionic conditions and temperatures was evaluated by steady-state probe-partitioning Förster resonance energy transfer (SP-FRET).³⁶ 0.3 mol % each of labeled cholesterol and PIP₂ were doped in LUVs, and the FRET efficiency was determined by exciting the LUVs at 490 nm and measuring the donor (TopFluor Cholesterol) fluorescence intensity ratio in the presence (I_{DA}) and the absence (I_D) of acceptor (BODIPY TMR-PIP₂) as the overall PIP₂ and DChol mole fractions were held constant.

Dynamic Light Scattering for LUV Size Distribution

The size distributions of PIP₂-containing LUVs were determined by measuring the autocorrelation of scattering intensity at a wavelength of 782.4 nm using a DynaPro99 instrument (Wyatt, former Protein Solutions) with a 3-window black quartz cuvette (Hellma, Kent, UK). The sample volume was 50 μ L.

Laurdan Generalized Polarization

0.3 mol % Laurdan dye was incorporated into LUVs during lipid film preparation. The order parameter of the bilayer was quantified by the fluorescence intensity at 440 and 490 nm using the Generalized Polarization (GP) function:³⁷

$$GP_{ex} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (2)$$

The emission profiles for the chosen lipid compositions were not sensitive to different excitation wavelengths from 340 to 360 nm.³⁸ The excitation wavelength is set at 360 nm for a better signal-to-noise ratio.

RESULTS

PIP₂-Mediated Inhibition of NtGSN Severing Activity Is Sensitive to Lipid Phase-Demixing

To fine-tune the lateral organization of the lipids, we followed the lipid phase diagram of a ternary lipid mixture composed of DOPC/DPPC/Chol.³⁴ An important assumption is that replacing DOPC with DO-PIP₂ at a low lipid mole fraction does not alter the feature of the phase diagram. This assumption was supported by the Laurdan GP measurements. As Laurdan has been shown to partition equally well in both liquid-ordered (L_o) and liquid-disordered (L_d) phases,³⁹ our assumption was supported by the fact that Laurdan emission spectra remained unchanged before and after replacing 5 mol % DOPC with DO-PIP₂ in LUV D (data not shown). The two spectra were nearly identical at 37 °C and below. A subtle difference was only found at a temperature above 45 °C, suggesting that the presence of minor amounts of PIP₂ might affect the phase transition temperature. Such a potential change at nonphysiologically elevated temperature however does not affect our conclusion, as all following comparisons between different LUVs were made at a room temperature. Here we assume PIP₂ distributes uniformly within the separated domains, but we cannot rule out the possibility that PIP₂ forms nanosized domains through the stabilization of cholesterol as reported elsewhere.⁴⁰

As a first step, we examined whether PIP₂-mediated inhibition of NtGSN was any different in the presence or absence of L_o/L_d phase separation (LUV A or B respectively) at a constant PIP₂ level. Studied by an actin-severing assay, the severing inhibition of NtGSN induced by LUV A and LUV B is significantly different even when both vesicle types contained 15 mol % PIP₂ (Figure 1A,B). In the presence of cholesterol-dependent phase separation, PIP₂ was presumably concentrated in the L_d phase as suggested by earlier studies.³⁵ The contrast between LUV A and LUV B shows that PIP₂-NtGSN interaction is sensitive not only to the global concentration, but also to the local concentrations of PIP₂.

Reducing PIP₂ mole fraction from 15 to 5 mol % under the same demixed condition as in LUV A significantly decreases but does not eliminate the capacity of PIP₂ to inhibit NtGSN (Figure 1A,C). The inhibition capability of PIP₂ in LUV C which contains a larger fraction of the L_d phase is restored by replacing 10 mol % DOPC with DPPC. An enhanced phase separation is expected following the lever rule in a phase diagram, as the tie lines of this specific system were characterized previously⁴¹ (Figure 1E). PIP₂ in LUV D is therefore expected to be further concentrated in the L_d domains with a smaller area fraction compared to that in LUV C. The difference between the lateral organization of LUV C and LUV D is reflected in their different abilities to inhibit NtGSN-mediated actin-severing (Figure 1C,D).

The inhibition of NtGSN using different PIP₂-containing LUVs is further quantified by the changes in the initial depolymerization rate of actin (Figure 2A,B). The normalized severing activities reflect the ratio between free and bound NtGSN. The fact that PIP₂ concentration is 2–3 orders of magnitude higher than NtGSN concentrations allows one to deduce the

membrane dissociation constants (Figure 2C,D). The measurements performed at 0.2 mM Ca^{2+} are similar to those at 1 mM EGTA and are therefore only summarized in Figure 2C,D. The finding that the addition of Ca^{2+} did not have a large effect on PIP₂ in inhibiting NtGSN enabled performing a similar experiment with full-length gelsolin, which requires micromolar Ca^{2+} for its activity. PIP₂ incorporated into a phase demixed vesicles like LUV A was much more effective than an equal mole fraction and total concentration of PIP₂ in the single phase LUV B in inhibiting full-length GSN (Figure 2E), confirming that the results with NtGSN can be extended to full-length gelsolin, and not only to Ca^{2+} -insensitive N-terminal gelsolin fragments such as those formed *in vivo* by activation of caspase.

Effects of Divalent Cations on LUV Sizes and Lipid Phase-Partitioning

Potential changes in vesicle structure induced by divalent cations were investigated by dynamic light scattering (DLS) and fluorescence resonance energy transfer (FRET). LUV D was chosen for these studies because its PIP₂ content is at a physiologically relevant mole fraction, and yet the severing inhibition by LUV D is significant at room temperature. Cation-induced vesicle aggregation was examined by DLS at different Ca^{2+} and Mg^{2+} concentrations, respectively (Figure 3A). The addition of Ca^{2+} did not lead to a significant change in LUV D vesicle sizes throughout all Ca^{2+} concentrations tested in the presence of 150 mM KCl. Mg^{2+} induces significant aggregation of the vesicles at above millimolar concentrations with more than 95% mass of the vesicles present in an aggregated form. Divalent cation-induced aggregation of LUV D was also evaluated at different temperatures since LUV D was used for a temperature-dependent study of gelsolin inhibition. LUV D does not aggregate in an F-buffer at most temperatures tested (Figure 3A, inset). Very few large aggregates with negligible mass fractions were found at temperatures higher than 45 °C. Lowering the temperature reverses the formation of vesicle aggregation. A similar but more drastic effect was observed using PIP₂ micelles instead of vesicles.

Divalent cation-dependent changes in phase partitioning of PIP₂ and DChol were investigated using the corresponding fluorescent analogues by SP-FRET as shown in Figure 3B. The energy transfer efficiency decreases significantly as Ca^{2+} concentration increases, suggesting that Ca^{2+} promotes further demixing between labeled PIP₂ and cholesterol in a phase-demixed membrane. In contrast, the decrease in energy transfer efficiency induced by Mg^{2+} is less discernible, and the presence of 2 mM Mg^{2+} in an F-buffer suppresses Ca^{2+} -induced changes in energy transfer efficiency. The measured FRET efficiency in LUV D increases with rising temperature (Figure 3B, inset), and it follows the phase transition of the membrane as shown by Laurdan GP (Figure 4C).

Inhibition of Severing Activity Induced by Demixed LUVs Is Temperature-Dependent

In order to verify the hypothesis that the inhibition of GSN severing activity is subject to changes in PIP₂ local concentration, the severing inhibition of NtGSN induced by LUV D at 54 μM PIP₂ was examined at different temperatures between 11 and 51 °C. Because cholesterol-mediated phase demixing is temperature dependent, LUV D at low temperature should be maximally demixed and become fully mixed at high temperatures.⁴² The initial actin disassembly rates in the absence of LUV D were first studied at different temperatures to set up the upper and lower limit of severing activities with and without NtGSN,

respectively. The temperature-dependent inhibition of NtGSN by phase-demixed LUV D was then investigated at different temperatures (Figure 4A). LUV D nearly completely inhibits NtGSN activity at 11 °C and LUV D-dependent inhibition of NtGSN declines significantly with increasing temperature as shown in Figure 4B. To test if the inhibition of NtGSN by PIP₂ in a demixed vesicle is reversed when the L_o/L_d phases mix at higher temperatures, gelsolin was first inactivated by incubation for 4 min at 11 °C with LUV D, followed by a 4 min incubation at 51 °C. As a control, the severing activity is compared to that of a sample incubated at 51 °C for 8 min. The inhibition of severing activity by LUV D at 11 °C is fully restored by incubating at a higher temperature. This result suggests that the severing activity of gelsolin can be rapidly switched on and off as PIP₂-containing membranes transform between uniform and demixed states. The temperature-dependent changes in inhibition are due to differences in the lipids and not the proteins, because in contrast to the effects of temperature on LUV D, the severing inhibition induced by PIP₂ micelles with similar inhibiting capability does not change with increasing temperature (Figure 4B).

Similar to the data analysis performed in Figure 2C,D, the temperature-dependence changes in NtGSN severing activity are converted into association equilibrium constants (K_A) as a function of temperature. The change in free energy upon binding to PIP₂-containing LUV D can be calculated from the relation:

$$\Delta G^\circ = -RT \ln K_A \quad (3)$$

The fact that $\ln K_A$ is linearly dependent on $1/T$ (Figure 4B, inset) allows one to extract the thermodynamic parameters ΔH° and ΔS° with a van't Hoff equation:

$$\ln K_A = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (4)$$

The ΔG° is -5.7 kcal/mol while ΔH° and ΔS° are -17.0 kcal/mol and -36.2 cal/mol·K, respectively.

Fraction of Severing Inhibition Correlates with Membrane Order

The temperature-dependent changes of order parameters in LUV D were investigated using Laurdan GP. Successful incorporation of Laurdan is confirmed by showing that pure DPPC LUVs have a melting temperature at about 40 °C^{37,38} (Figure 4C). The membrane order of LUV D, with or without PIP₂, does not vary with the presence of divalent cations as used in the F-buffer and are therefore pooled together. The phase transition in LUV D, which corresponds to a mixing of L_o and L_d phases, is less sharp compared to a gel-to-liquid phase transition in pure DPPC LUVs. The incorporation of 5 mol % PIP₂ further smooths out the phase transition in LUV D (Figure 4C). The almost linear temperature-dependent changes in Laurdan GP correlate well with the temperature-dependent changes in the severing inhibition of NtGSN (Figure 4D).

DISCUSSION

Varying the lateral distribution of PIP₂ in sub-micron-sized mixed lipid vesicles has a strong effect on the ability of PIP₂ to inhibit gelsolin, one of the most abundant PIP₂-regulated cytoskeletal proteins. The results suggest that it is the local PIP₂ concentration in nanoscale domains, rather than the overall PIP₂ mole fraction, that determines the interaction of PIP₂ with its target binding proteins. Equivalently, this result suggests that PIP₂-binding proteins can be regulated by locally perturbing the PIP₂ lateral organization without changing the overall lipid composition.

The observed rate constant k_1 from single exponential fitting reflects an apparent rate constant of actin depolymerization, which is linearly proportional to the pointed end concentration and is about the same as the NtGSN concentration. The fact that the fluorescence time courses can be fit by single exponential decay suggests that the association and severing of NtGSN are not captured with this setup and are happening within the first few seconds after mixing. This argument is in agreement with the association and severing kinetics of GSN determined from stopped flow experiments,⁴³ in which the determined association, dissociation, and severing rate constants are $1.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, 0.4 s^{-1} , and 0.27 s^{-1} , respectively. While similar values were reported with the N-terminal half of gelsolin,⁴⁴ it is estimated that 63% of the severing events happen within the first 6 s after F-actin is mixed with 31 nM NtGSN.

The temperature variation experiments used to test the effects of lipid demixing in vesicles of constant composition can also be used to extract information about the thermodynamics of actin monomer dissociation from the pointed end of gelsolin-capped filaments. The extracted rate constants from single exponential decays at different temperatures allow us to evaluate the energy barrier for an actin monomer to dissociate from F-actin pointed ends based on the classic Arrhenius plot in which the activation energy is determined by the slope from a linear fit.

$$\ln k_1 = -\frac{E_a}{R} \frac{1}{T} + \ln A \quad (5)$$

The determined activation energy in the absence of NtGSN is 6.3 kcal/mol, similar to the value of 6.5 kcal/mol that was reported in earlier studies.⁴⁵ Its physical meaning is however difficult to evaluate because k_1 also depends on the filament length distribution, which follows an exponential distribution.^{46,47} Interestingly, the slope of the Arrhenius plot does not change in the presence of NtGSN (data not shown). This result is consistent with previous conclusions that gelsolin facilitates actin disassembly by generating more free pointed ends through severing, but not by facilitating monomer dissociation, at least for filament lengths where the fluorescence difference between G and F-actin is maximal.

The degrees of gelsolin inhibition by demixed or uniphase unilamellar vesicles with the same PIP₂ mole fractions can be very different. The inhibition is very limited when PIP₂ is evenly distributed in single phase vesicles such as LUV B. An enhanced PIP₂-NtGSN interaction without varying PIP₂ mole fraction can be achieved by introducing L_o/L_d phase

separation in the membrane. Phase-demixed vesicles at the same total PIP₂ level such as LUV A are significantly more effective in NtGSN inhibition (Figure 2A). The corresponding K_D for PIP₂ in LUV A in binding to NtGSN is 15 μ M and is about 30 fold lower than that in LUV B, whose K_d (430 μ M) falls out of the range in which the interactions are likely to be physiologically relevant (Figure 2C). The magnitude of the differences in binding of gelsolin to PIP₂ in mixed or demixed membranes is large enough so that actin binding and severing activity at the cytosol/membrane interface can be switched on or off simply by changes in the structuring of the membrane bilayer.

When the PIP₂ mole fraction is lowered from 15 mol % (LUV A) to 5 mol % (LUV C), the effective K_D increases by an order of magnitude. This result is reasonable since the dissociation constant is a function of membrane surface potential.^{48,49} A more than 10-fold decrease in binding affinity is expected since the surface potential is proportional to the surface charge density at a high surface potential regime.⁵⁰ The impaired binding due to a lowered PIP₂ mole fraction can be rescued by increasing the mole fraction of saturated lipids in the vesicles (Figure 1D) without changing the total amount of PIP₂ in the vesicle. As shown in Figure 1E, LUV D is closer to the phase boundary than LUV C, and this difference implies a more condensed packing of PIP₂ since the area fraction of the L_d phase is expected to decrease based on the lever rule. Therefore, the interactions between PIP₂ and PIP₂-binding proteins can be regulated through a perturbation in PIP₂ lateral organization without varying the overall PIP₂ concentration at a near-physiological condition.

Since Ca²⁺ also causes local concentration of PIP₂ into 100 nm scale clusters,^{12,35,51} and affects the phase partitioning of PIP₂ (unpublished data) in model membranes, the possible effect of Ca²⁺ on PIP₂-NtGSN interaction was also investigated. However, the addition of Ca²⁺ only slightly increases the affinity of PIP₂ for NtGSN in all cases tested (Figure 2C,D). A potential explanation is that the Ca²⁺-induced clusters of PIP₂ have a lower net charge due to charge neutralization.⁵²

Since Ca²⁺ and Mg²⁺ are known to induce PIP₂ micelle aggregation⁵³ and promote vesicle fusion of anionic lipid-containing LUVs,^{54–58} it is therefore important to test whether PIP₂-containing vesicles aggregate in the presence of divalent cations. LUV D does not aggregate in F-buffer, although LUV D is prone to aggregate in the presence of millimolar Mg²⁺. This result suggests that competitive binding of Ca²⁺ to PIP₂ inhibits Mg²⁺-induced aggregation since Ca²⁺ does not promote vesicle aggregation at an even higher concentration (Figure 3A). The fact that the formation of large aggregates of LUV D at higher temperature can be reversed by lowering the temperature suggests that it might be a result of enhanced electrostatics between divalent cations and PIP₂ due to a lowered dielectric constant at higher temperatures.⁵⁹

The effect of Ca²⁺ on PIP₂ lateral organization in a phase-demixed membrane was examined in more detail using SP-FRET in order to determine the extent to which PIP₂ segregates from other lipids such as cholesterol in this study. The fact that FRET efficiencies decrease with increasing Ca²⁺ concentration (Figure 3B) suggests that PIP₂ molecules are only partially segregated from cholesterol upon L_o/L_d phase demixing in the absence of Ca²⁺. The fact that PIP₂ and cholesterol are partially segregated in the absence of Ca²⁺ is

confirmed using SP-FRET by showing the FRET efficiency increases with rising temperature (Figure 3B, inset). Ca^{2+} promotes further demixing of PIP_2 from cholesterol at a millimolar concentration, whereas Mg^{2+} has a very limited effect. A Ca^{2+} -induced change in FRET efficiency was inhibited by Mg^{2+} as shown in Figure 3B, and Mg^{2+} has been suggested to compete with Ca^{2+} in binding to PIP_2 with similar binding affinities.¹² These results together explain why the presence of Ca^{2+} has a minimal effect on PIP_2 -NtGSN interactions.

The observation that PIP_2 -GSN interaction is modulated by lipid lateral organization is further confirmed by varying the temperature while using phase-demixed LUVs. The L_o/L_d phase transition temperature of LUV D is estimated in between 25 and 30 °C,⁴² which is spanned by the temperature range shown in Figure 4. The result that severing inhibition by LUV D is reversible and temperature-dependent supports our hypothesis. Performing the same experiments with pure PIP_2 micelles served an important control, which rules out the possibility that the interaction between PIP_2 and NtGSN was weakened by the increased thermal fluctuation at a higher temperature. PIP_2 micelles at 25 μM PIP_2 is just enough to fully inhibit NtGSN at room temperature²⁴ and was confirmed with our study (data not shown), and the inhibition efficiency remains unchanged throughout the temperature range tested. Therefore, an increased thermal fluctuation does not account for the impaired interaction between NtGSN and PIP_2 -containing LUV D.

In order to relate the temperature dependence in severing inhibition with the phase behavior of the lipids, Laurdan GP was used to probe the changes in the physical states of the membrane. The phase transition measured by Laurdan GP is less discernible in LUV D, either with or without PIP_2 , compared to that in pure DPPC LUVs. The incorporation of PIP_2 further smoothed out the phase transition, which then appears to vary linearly with the temperature change. A good correlation between severing activity and the membrane order of LUV D once again supports the hypothesis that PIP_2 -NtGSN interaction is modulated by the lateral distribution of the PIP_2 in the membrane.

CONCLUSION

The results shown here demonstrate that the interaction between PIP_2 and NtGSN is sensitive to local concentrations of PIP_2 and is therefore subject to changes in lipid lateral organization at a physiological ionic condition. Cholesterol-dependent phase-demixing greatly enhances PIP_2 -NtGSN interaction at a fixed PIP_2 mole fraction. The fact that the inhibition decreases with increasing temperature correlates well with the temperature-dependent phase transition of a demixed lipid bilayer.

The evidence that there might be an additional level of regulation in gelsolin-mediated actin remodeling that is mediated by lipid lateral organization suggests that the similar regulation might occur for other lipid-binding proteins. The same approach was adopted to investigate the membrane crowding effect of a PIP_2 -binding ENTH domain in phase-demixed GUVs.⁶⁰ Similarly, it was demonstrated that the binding affinity of PTEN to PI5P- and PIP_2 -containing GUVs increased significantly in the presence of cholesterol-promoted phase separation,⁴⁰ although the mechanism by which lipids phase-separated was different and

more likely driven by hydrogen bonding. Other examples involve the annexin protein family. While the *in vitro* experiments employing solid-supported model membranes do not reveal a direct interaction between annexin A2 and cholesterol,⁶¹ the affinity of annexin A2 toward PS or PIP₂ in a PC background increased significantly upon the addition of cholesterol in model membranes.^{62–64} Similar observations were found with annexin A1.⁶⁵ Although it is still under debate whether annexins interact with cholesterol directly, a Monte Carlo simulation suggested that the protein–protein interaction of annexin promotes the coarsening of PS-rich microdomains only if cholesterol-mediated phase segregation is present.⁶⁶

Whether cholesterol modulates the regulation of gelsolin activity at a cellular level was not addressed in this study and is therefore still an open question. Some hints from earlier studies suggest that cholesterol modulates the membrane binding affinity and therefore intracellular localization of annexin A6.⁶⁷ Furthermore, a defect in cholesterol regulation at a cellular level has many biological consequences. A dysregulation of cholesterol transport associated with an abnormal cellular distribution of annexin A6 is commonly found in the human lysosomal storage disease Niemann-Pick type C disease.⁶⁸ These examples together with what we have demonstrated in this study strongly suggest that lipid lateral structure might serve as an additional mode of protein regulation that alters cortical actin assembly.

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ABBREVIATIONS

PIP2	phosphatidylinositol 4,5-bisphosphate
NtGSN	N-terminal fragment of gelsolin
LUVs	large unilamellar vesicles
DOPC	dioleoylphosphatidylcholine
DPPC	dipalmitoyl-phosphatidylcholine
DChol	dihydrocholesterol

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Figure 1. Severing inhibition induced by PIP₂-containing LUVs. (A–D) Representative intensity profiles of actin depolymerization at 31 nM NtGSN and 1 mM EGTA at various concentrations of PIP₂-containing LUVs. The single exponential fitting is shown in yellow as demonstrated in panel A. The corresponding lipid compositions of LUV A, B, C, and D are indicated in panel E. (E) Phase diagram of ternary lipid mixtures and LUVs with indicated lipid compositions. Image (E) is adapted with permission from *Biophys. J.* **2003**, *85*, 3074–3083. Copyright 2003 The Biophysical Society. Lipid composition (PIP₂/DOPC/

DChol/DPPC) for LUV A = 15/10/30/45; LUV B = 15/80/0/0; LUV C = 5/20/30/45; LUV D = 5/10/30/55.

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Figure 2.

PIP₂-dependent inhibition of NtGSN and the corresponding affinity constants quantified from the initial rate of depolymerization. (A and B) PIP₂ concentration-dependence of different LUVs in NtGSN severing inhibition at 31 nM NtGSN and 1 mM EGTA at pH7.0. Experiments performed with 0.2 mM Ca²⁺ were similar to those performed at 1 mM EGTA and are therefore omitted. (C and D) The deduced association constants (mean ± S.D.) corresponding to the normalized severing activities. (E) Similar experiment performed with 20 nM full-length GSN using LUV A and B at 0.2 mM Ca²⁺ and pH7.0. Lipid composition (PIP₂/DOPC/DChol/DPPC) for LUV A = 15/10/30/45; LUV B = 15/80/0/0; LUV C = 5/20/30/45; LUV D = 5/10/30/55.

Figure 3.

The effects of divalent cations and temperature on the size of vesicles and the lateral organization of the membrane. (A) Concentration-dependent changes in vesicles sizes induced by Ca^{2+} and Mg^{2+} . The percentages indicate the mass fraction of the aggregations. (Inset) The size dependence of LUV D in an F-buffer measured from high to low temperatures. The data reported are mean $\pm$ S.D., $n=20$. (B) Concentration-dependent changes in SP-FRET efficiency of BODIPY TMR-PIP₂ and Topfluor-Chol induced by Ca^{2+} and Mg^{2+} . Ca^{2+} -induced changes in FRET efficiency are inhibited by the presence of 2 mM

Mg²⁺. (Inset) FRET efficiency of the same probe pair in LUV D increases with increasing temperature in an F-buffer. Lipid composition (PIP₂/DOPC/DChol/DPPC) for LUV D = 5/10/30/55.

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Figure 4.

Temperature-dependent severing inhibition induced by LUV D correlates with the changes in membrane order. (A) Severing inhibition of 31 nM NtGSN in the presence of LUV D at 54 μ M PIP₂ at various temperatures. The initial disassembly rates of actin in the absence of LUV D are fit by an Arrhenius equation as shown with the dotted lines. (B) Normalized severing activity of NtGSN inhibited by LUV D from panel A or by PIP₂ micelles at various temperatures. Gray circles represent data collected after an additional 4 min incubation at 51 °C. (Inset) NtGSN-PIP₂ interactions at different temperatures fit with the van't Hoff equation. (C) Temperature-dependent changes in Laurdan GP (λ_{ex} = 360 nm) of pure DPPC LUVs and LUV D with or without PIP₂ in an F-buffer. (D) The temperature-dependence of normalized severing inhibition, defined as the difference from 100% severing activity, correlates well with the changes in membrane order in LUV D. All data presented except

two baselines in panel A are mean values of three different samples, and the uncertainties are the standard deviations, not shown if they are smaller than the symbols. Lipid composition (PIP₂/DOPC/DChol/DPPC) for LUV D = 5/10/30/55.

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