Title

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Journal

Biophysical Journal, 120(7)

ISSN

0006-3495

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Publication Date

2021-04-01

DOI

10.1016/j.bpj.2020.09.017

Peer reviewed

Biophysical Journal
Article

Biophysical Society

Coupled membrane lipid miscibility and phosphotyrosine-driven protein condensation phase transitions

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ABSTRACT Lipid miscibility phase separation has long been considered to be a central element of cell membrane organization. More recently, protein condensation phase transitions, into three-dimensional droplets or in two-dimensional lattices on membrane surfaces, have emerged as another important organizational principle within cells. Here, we reconstitute the linker for activation of T cells (LAT):growth-factor-receptor-bound protein 2 (Grb2):son of sevenless (SOS) protein condensation on the surface of giant unilamellar vesicles capable of undergoing lipid phase separations. Our results indicate that the assembly of the protein condensate on the membrane surface can drive lipid phase separation. This phase transition occurs isothermally and is governed by tyrosine phosphorylation on LAT. Furthermore, we observe that the induced lipid phase separation drives localization of the SOS substrate, K-Ras, into the LAT:Grb2:SOS protein condensate.

SIGNIFICANCE Protein condensation phase transitions are emerging as important organizing principles in cells. One such condensate plays a key role in T cell receptor signaling. Immediately after receptor activation, multivalent phosphorylation of the adaptor protein linker for activation of T cells (LAT) at the plasma membrane leads to a networked assembly of a number of signaling proteins into a two-dimensional condensate on the membrane surface. In this study, we demonstrate that LAT condensates in reconstituted vesicles are sufficient to drive lipid phase separation. This lipid reorganization drives another key downstream signaling molecule, Ras, into the LAT condensates. These results show that the LAT condensation phase transition, which is actively controlled by phosphorylation reactions, extends its influence to control lipid phase separation in the underlying membrane.

INTRODUCTION

In 1973, shortly after the classic fluid mosaic description of cell membranes was published (1), a series of articles from Harden McConnell's lab described discovery of lateral phase separation in the lipids of cell membranes (2–6). Contemporary work from Sackmann and colleagues confirmed an intriguing heterogeneity in the organization of lipids in the fluid membrane (7). This phenomenon later developed into the lipid raft model of cell membranes, as articulated by Simons and Ikonen (8–10). The field of lipid rafts has since both flourished and attracted great controversy (11–14). Although lipid miscibility phase transitions are readily and spectacularly visualized in purified lipid membranes (Fig. 1 *A*; (15–17)), their unambiguous detection in living cells proved much more challenging (18–21). Reports of definitive observation are sparse (22), suggesting more complex behavior may prevail in most circumstances. There is evidence that cell membranes are poised near a miscibility phase transition (23), which naturally leads one to speculate that this may be actively controlled by the cell. However, a longstanding criticism of the lipid raft model questions how lipid phase separation

Submitted July 22, 2020, and accepted for publication September 17, 2020.

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Editor: Rohit Pappu.

https://doi.org/10.1016/j.bpj.2020.09.017

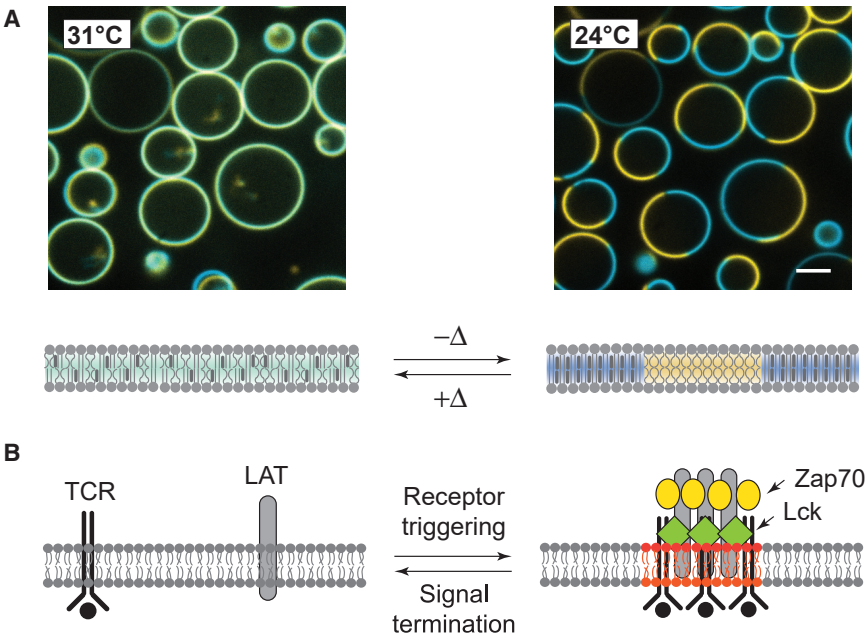

FIGURE 1 (*A*) Representative giant unilamellar vesicles (GUVs) showing temperature-dependent liquid-liquid phase separation. At 31°C, which is above the transition temperature T_{misc} of 29°C, the distribution of lipids is homogeneous across the membrane for 100% of the sample of ~100 vesicles. Below T_{misc} at 24°C (*right*), lipids compartmentalize into macroscopic domains for 99% of the sample of ~100 vesicles: the L_d domain (TR-DHPE; *yellow*) is enriched with unsaturated lipids, and the L_o with saturated lipids (OG-DHPE; *blue*). All GUV experiments are performed in buffer with matching osmolarity (50 mM Tris and 150 mM NaCl (pH 7.4)). Typical vesicle concentrations were ~0.2 mg/mL. Scale bars, 5 μm. The ensemble average temperature-dependent phase separation (n ~100 vesicles) is shown in Fig. 4 (*right panel*, *empty circles*). (*B*) In lipid raft theory, clusters of signaling proteins, such as the TCRs, are "carried" on ordered lipid domains to facilitate signal transduction.

could be controlled with the specificity required for biological functions, whereas the underlying interactions between lipids and cholesterol that enable the phase transition are rather nonspecific (24). Clearly proteins must play a commanding role controlling lipid phase separation in the physiological setting, but we have very limited mechanistic understanding of how this is actually achieved in specific cases (25).

One prominent example that captures this debate is the T cell receptor (TCR) signaling system (Fig. 1 *B*). TCR and a number of downstream proteins, including linker for activation of T cells (LAT), phospholipase C γ 1, and the Ras activator son of sevenless (SOS), form clusters on the membrane (26–32). Earlier studies using detergent-resistant membrane extraction have suggested that these molecules reside on lipid rafts (33–35). However, subsequent studies have failed to conclusively establish lipid rafts as the driving force for TCR-induced signaling clusters (36–38). Furthermore, it remains unclear how signaling activity—in the case of TCR, the receptor activation and tyrosine phosphorylation of downstream proteins including LAT—could trigger the lipid phase separation. This disconnect is further underscored by the fact that at physiological ligand densities (39), individual TCR are capable of triggering the entire signaling pathway without ever forming clusters themselves (40–44).

Modular binding interactions among proteins present another type of mediated molecular assembly process in cells (45,46). With sufficient multivalency, these interactions can lead to protein condensation phase transitions into three-dimensional droplets (47), sometimes called membraneless organelles, or two-dimensional assemblies on the membrane surface (48–51). Similar biomolecular condensates can also incorporate nucleic acids and play a role in transcription regulation (52,53).

It has recently been discovered that LAT can participate in a protein condensation phase transition in reconstituted membranes (48,49,54,55). LAT is a transmembrane scaffold protein that becomes phosphorylated at multiple tyrosines upon TCR activation. Three of the phosphotyrosines on LAT are canonical docking sites for the SH2 domain of growth-factor-receptor-bound protein 2 (Grb2), a cytosolic adaptor protein (56). Grb2 additionally has SH3 domains, which bind to the proline-rich domain of SOS, a guanine nucleotide exchange factor that activates Ras (57). A single SOS can associate with at least two Grb2 molecules, and these multivalent interactions result in an extended two-dimensional network assembly of LAT:Grb2:SOS on the membrane in a phosphorylation-dependent manner (58,59). This complex has been shown to play an important role in T cell signaling (60,61). The LAT:Grb2:SOS protein condensation phase transition is reversible, and because it is governed by tyrosine phosphorylation, it is directly under the control of competing kinase and phosphatase reactions in the TCR signaling system.

Here, we reconstitute the LAT:Grb2:SOS protein condensate from purified proteins on giant unilamellar vesicle (GUV) membranes that can undergo lipid phase separation. The LAT condensation induces lipid phase separation in GUVs, even above the miscibility temperature of the lipid composition. We additionally observe that protein condensation-induced lipid phase separation further directs spatial organization of the downstream membrane-bound molecule, K-Ras. These results illustrate how a protein

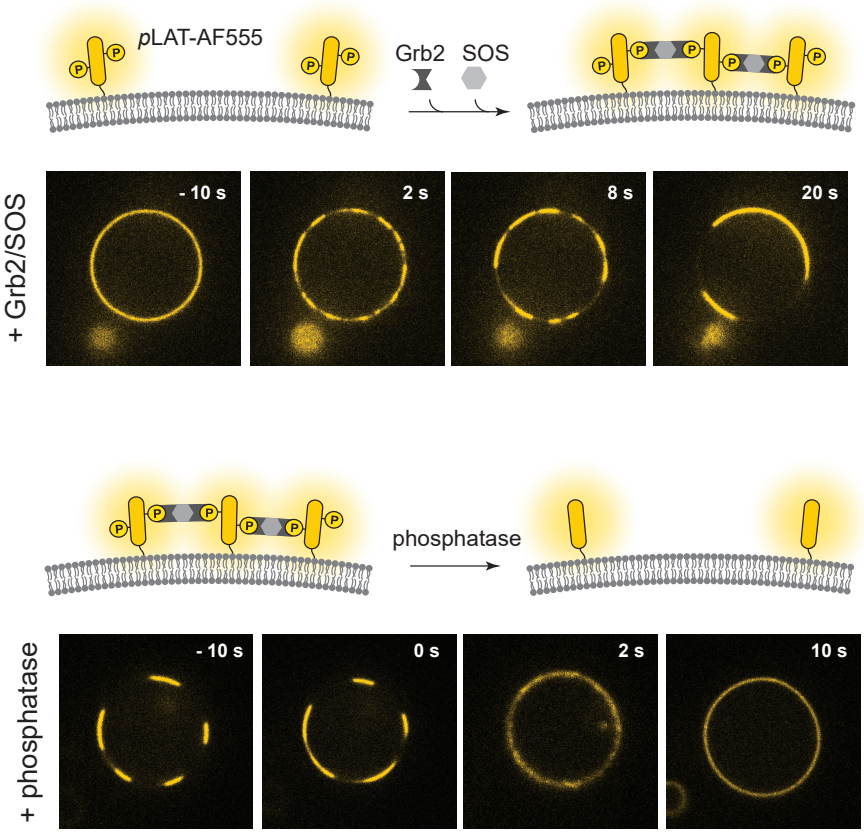

FIGURE 2 The LAT:Grb2:SOS protein condensate was reconstituted on GUVs. His-tagged *p*LAT is associated with the vesicles by chelating to Ni-nitrilotriacetic-acid lipids. The introduction of a 1.2-μM, full-length Grb2 and 0.8 μM proline-rich domain of SOS results in extended networks of LAT condensate that is visualized by the AF555 fluorescence in the confocal microscopy (*top*). The LAT:Grb2:SOS assembly can be reversed by dephosphorylation of LAT by phosphatase (5 μM YopH) (*bottom*). Under the experimental conditions used, LAT showed condensation on most (>95%) of the vesicles and reversal by phosphatase (two independent experiments, n ~50 vesicles). Scale bars, 5 μm. To see this figure in color, go online.

condensation phase transition, which is directly under control of a specific signaling system, can drive miscibility phase transitions in the underlying lipid membrane.

RESULTS

LAT:Grb2:SOS condensation on vesicles drives lipid phase separation

Phosphotyrosine-mediated LAT:Grb2:SOS condensates were reconstituted in GUVs with lipid composition that can undergo lipid phase separation. The cytoplasmic domain of LAT was purified with an N-terminal His_6 tag and labeled with Alexa Fluor 555 (AF555) at Cys146 via maleimide-thiol chemistry. LAT was phosphorylated by the kinase domain of Hck in solution. Then, phosphorylated LAT (*p*LAT) was linked to the membrane by the binding of the His_6 tag to the Ni-nitrilotriacetic-acid lipids in the membrane. This membrane-linked *p*LAT exhibits free lateral diffusion and remains monomeric before any assembly (48,49). The addition of full-length Grb2 and the proline-rich domains of SOS leads to the networked condensation of LAT:Grb2:SOS on the membrane surface of GUVs, as shown in Fig. 2 (*top row*). Here, the condensates are visualized as concentrated regions of *p*LAT-AF555 fluorescence on GUVs by confocal microscopy. This condensate is mediated by tyrosine phosphorylation on LAT and is reversible (Fig. 2, *bottom row*). The rapid (<10 s) dispersion of the condensed structure upon phosphatase (YopH) addition indicates that the individual Grb2:*p*LAT bonds must be highly dynamic and offer little protection from solution phosphatases. Incidentally, the membrane-linked phosphatase CD45 has been reported to be excluded from LAT condensates, possibly providing some degree of positive feedback with respect to this phosphatase (48). These basic features of LAT condensates on GUVs are similar to LAT condensates on supported membranes (48,49,55); detailed characterizations on supported membranes show that LAT condensates form simultaneously across the membrane and coalesce into larger domains over time. The faster diffusivity in GUVs led to larger domains that simplify the comparison with the lipid organization.

We next examined how the lipid phase transition behavior of GUVs is perturbed by the LAT condensate. GUVs that are composed of a ternary mixture of saturated lipids, unsaturated lipids, and sterols (in a roughly 1:1:1 ratio) exhibit temperature-dependent miscibility phase separation. Below the miscibility transition temperature (T_{misc}), the vesicles separate into coexisting liquid-ordered (L_o) and liquid-disordered (L_d) regions (16,62). As a crude guideline, the L_o region is rich in saturated phosphatidylcholine lipids such as dipalmitoylphosphatidylcholine (DPPC), whereas L_d is

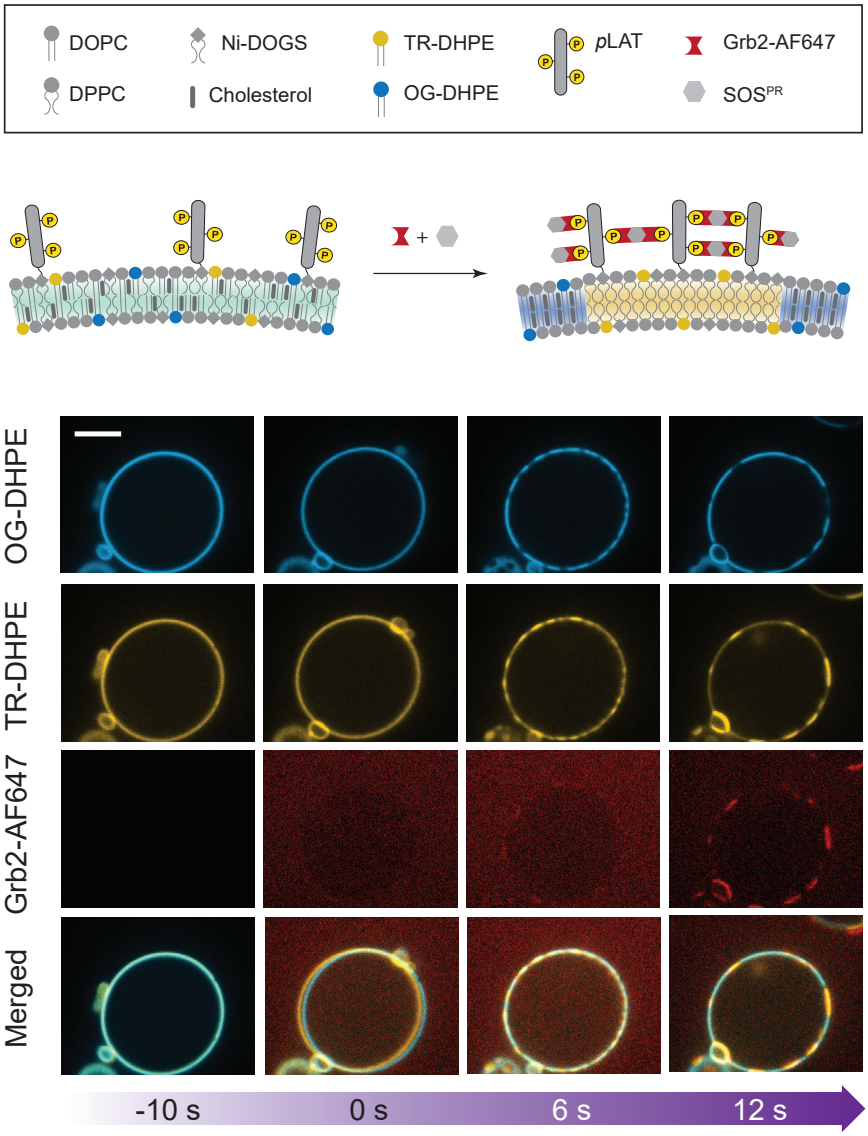

FIGURE 3 An example of a video showing the LAT:Grb2:SOS condensate-induced lipid phase separation on GUVs. Starting with a temperature (31°C) above its T_{misc} (29°C), the lipids are initially spatially homogeneous. As the proteins assembled (visualized by doping unlabeled Grb2 with 1% Grb2-AF647), the lipids undergo liquid-liquid phase transition. OG-DHPE and TR-DHPE mark the L_o and L_d regions, respectively. All vesicles that were capable of phase separation transitioned within 30 s. The ensemble average data for the temperature-dependent phase separation ($n \sim 100$ vesicles) are shown in Fig. 4 (*right panel*, *red circles*). Scale bars, 5 μm. To see this figure in color, go online.

rich in unsaturated phospholipids such as dioleoylphosphatidylcholine (DOPC) (63). For our experiments, GUVs composed of 29.2% DOPC, 33.2% DPPC, 33.3% cholesterol, 4% Ni-DOGS, 0.1% Texas-Red (TR)-*N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (DHPE), and 0.2% Oregon-Green (OG)-DHPE were used. This composition is an approximation of the well-characterized equimolar mixture of DOPC, DPPC, and cholesterol, and the observed T_{misc} is also close to the reported value, 29°C (62). Even though it is not critical into which lipid phase LAT partitions in our experiments, the full-length protein has been shown to partition into clusters without lipid raft makers (glycosylphosphatidylinositol (GPI) anchors) in live cells (38)—suggesting that LAT does not partition into the L_o-like phase in cells. In our experiments, because the Ni-DOGS chain is unsaturated (18:1-18:1), Ni-chelated *p*LAT is expected to partition into the L_d region. This is confirmed by its colocalization with TR-DHPE, which is a well-established reporter of the L_d phase (16). On the other hand, TR and OG fluorescence exclude each other upon phase separation, indicating that OG-DHPE partitions into the L_o phase (Fig. 1 *A*).

First, we examined whether LAT condensation could induce phase transitions in initially uniform vesicles near the T_{misc}. The experiment is shown in Fig. 3. In the imaging chamber maintained at 31°C, the *p*LAT-associated vesicle membranes exhibit a homogeneous distribution of fluorescent markers (TR-DHPE and OG-DHPE), as expected because this temperature is slightly above the T_{misc} of 29°C. The addition of Grb2-AF647 and SOS triggers a rapid LAT:Grb2:SOS condensation on the membrane surface, which is readily visualized by the appearance of concentrated regions of 647-nm fluorescence, tracking Grb2. This

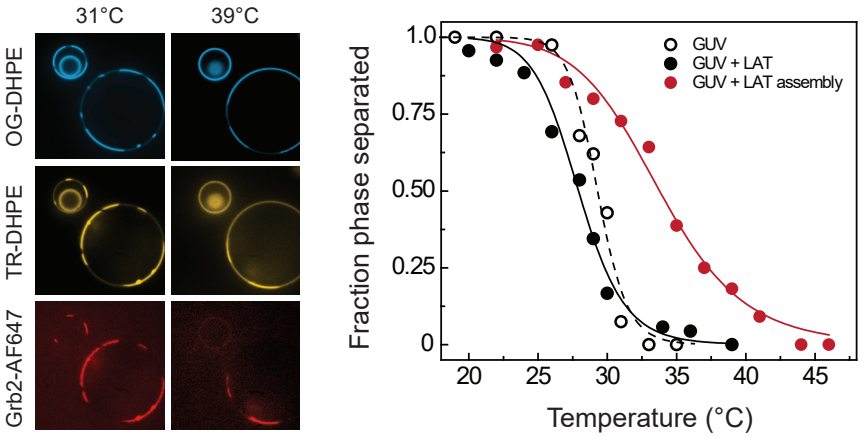

FIGURE 4 (*Left*) At 31°C, the GUVs associated with LAT:Grb2:SOS clusters are phase separated. Note that the smallest vesicle, invaginated within a larger vesicle, is inaccessible to the proteins and remains homogeneous. At 39°C, Grb2 has dissociated from the smaller vesicle, which became homogeneous. For the larger vesicle on which the protein condensate remains, the lipid of the phase separation also remains. Scale bars, 2 μm. (*Right*) The miscibility transition temperatures (T_{misc}s) were measured for bare GUVs (T_{misc} = 29.3 ± 0.5°C), GUVs with LAT (T_{misc} = 27.8 ± 0.4°C), and GUVs with the LAT condensate (T_{misc} = 33.9 ± 0.5°C) by counting the fraction of phase-separated vesicles as a function of temperature then fitting them to the logistic function. Although the difference in T_{misc} between bare GUVs and LAT-associated GUVs are minimal, it is increased significantly in the presence of the protein assembly. The data primarily reflect temperature-dependent LAT:Grb2:SOS interactions rather than GUV phase separation because the protein assembly becomes unstable at high temperatures and dissociates from the vesicles. However, hypothetically, stable LAT:Grb2:SOS interactions would further increase the apparent T_{misc}. The GUV counts are shown in Table S1. To see this figure in color, go online.

is accompanied by a clear partitioning of TR-DHPE (*yellow*) and OG-DHPE (*blue*), indicating a miscibility phase transition within the lipids has also occurred, although under isothermal conditions here. The LAT condensate is coincident with the L_o region marked by TR-DHPE, whereas the L_d region, visualized by OG-DHPE, is excluded from LAT.

LAT condensation increases the apparent miscibility temperature of lipid phase separation

The presence of LAT condensation alters the temperature-dependent phase separation behavior of the vesicles in equilibrium (Fig. 4). In this experiment, the temperature of the imaging chamber was increased gradually at a rate of ~1°C/min and held at each temperature data point for 2 min. Then, multichannel confocal images of a population ($n \sim 100$) of vesicles were obtained. The chamber was cooled back to 20°C at the same rate. The number of phase-separated vesicles were counted at each temperature point. All observations were the same regardless of the direction of the temperature ramp, indicating that all processes, including protein assembly and lipid phase separation, are reversible. Bare GUVs (*empty black circles*) show a T_{misc} of 29°C. With *p*LAT associated with the vesicles (*solid black circles*), T_{misc} is shifted slightly but remains essentially the same at 28°C. When the LAT condensate is formed by the addition of Grb2 and SOS, however, the apparent T_{misc} is increased to 34°C (*red solid circles*). This is consistent with the previous experiment in which the lipid phase separation is driven by the protein condensate at a temperature at which it would otherwise be homogeneous.

The apparent ΔT_{misc} of 5°C in the presence of the protein condensate is not actually a shift in the lipid T_{misc}. Rather, the protein condensate itself becomes unstable at higher temperatures, and Grb2 and SOS are released from the vesicle surfaces. This can be seen in Fig. 4, bottom right: at 39°C, the Grb2-AF647 fluorescence is not redistributed on the membranes but rather reduced overall because it was lost to the solution. The fluorescence signal is recovered when the temperature is lowered, indicating that the protein condensation is also a reversible, temperature-dependent process. As long as the protein condensate is present, vesicles remained phase separated with the L_d region templating the protein condensate (Fig. 4). This suggests that the actual ΔT_{misc} is greater than the apparent value of 5°C and probably lies outside the experimentally accessible temperature range in which both the GUV phase separation and LAT:Grb2:SOS condensate can be observed.

LAT-condensation-induced lipid phase separation drives K-Ras into the condensates

The ability for condensates to induce lipid phase separation raises a potential mechanism to spatially reorganize downstream membrane-bound proteins. K-Ras is a small GTPase and a substrate of SOS, and an SOS-catalyzed nucleotide exchange from its GDP-bound state to its GTP-bound state triggers downstream signal activation. The various Ras isoforms serve as hubs for signaling pathways, such as phosphoinositide 3-kinases (PI3K) and mitogen-activated protein kinase (MAPK), and Ras misregulation is among the most common causes of cancer (64,65). Native K-Ras is localized to the membrane by a farnesyl lipid modification as well as electrostatic interactions between its positively charged region and anionic phospholipids in cellular membranes (66). Therefore, the organization of lipids is expected to play an important role in determining the location of K-Ras. Previous studies have shown that K-Ras partitions to the L_d region on GUVs largely because of the highly branched farnesyl anchor (67). Therefore, we anticipated that K-Ras may similarly be directed by the lipid phase

separation induced by the LAT:Grb2:SOS condensate. To examine this, enhanced green fluorescent protein (eGFP)-labeled full-length K-Ras with its native membrane anchor, including both the farnesylation and methylation of the terminal cysteine (68,69) (20 nM final concentration), was introduced into GUVs of similar composition as in the previous experiments but with 10% anionic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) lipids (final composition: 19.3% DOPC, 33.3% DPPC, 33.3% cholesterol, 10% DOPS, 4% Ni-DOGS, and 0.1% TR-DHPE). The negatively charged lipids are necessary for the stable association of K-Ras to the membrane (69–71). The bottom panel of Fig. 5 shows that before the introduction of Grb2 and SOS, eGFP-K-Ras (*blue*) as well as TR-DHPE (*yellow*) are initially distributed homogeneously on the vesicles. After Grb2 (*red*) and SOS are added, the lipid membrane becomes phase separated as the protein assemblies form on its surface. K-Ras, LAT:Grb2:SOS, and TR are observed to partition together in the L_d region. The enrichment of K-Ras within the condensates was ~5-fold compared with molecules outside of the condensates (the apparent partition coefficient $K = \frac{Ras_{in}}{Ras_{out}} \sim 4.7 \pm 0.4$, where $\pm$ denotes SEM). Coupling of the lipid miscibility phase separation to the LAT:Grb2:SOS protein condensation localizes K-Ras with the condensate. Because K-Ras does not colocalize with the protein condensate on supported lipid bilayers that are incapable of phase transitions (Fig. S1), its partitioning on GUVs is likely to be driven by its anchor participating in the lipid phase transition. This phase transition and subsequent protein colocalization between SOS and K-Ras occurs isothermally and under the control of tyrosine phosphorylation reactions.

DISCUSSION

In summary, we have reconstituted the T cell signaling condensate, LAT:Grb2:SOS, on vesicles capable of undergoing liquid-liquid miscibility phase transitions. We observed that the formation of protein condensate can drive the lipid phase transition under isothermal conditions, redistributing lipids in a signal-dependent manner. Furthermore, we have shown that K-Ras, which does not directly participate in the LAT:Grb2:SOS condensation, nonetheless colocalizes with the condensate through its sensitivity to the lipid environment. Lipid phase separation can also be induced by actin polymerization (72,73) and lipid cross-linking by cholera toxin (74). Unique to the observations reported here, however, is that the LAT:Grb2:SOS protein condensation occurs immediately downstream of TCR activation, and as a direct result of ZAP70 kinase activation on triggered TCRs (28,75). ZAP70 is an Syk family kinase that exhibits a distinctive substrate specificity that is orthogonal to that of other kinases in the TCR signaling system, and strongly favors phosphorylation of the specific tyrosine residues on LAT that are involved in the LAT condensate (76). In this way, the LAT condensation phase transition is selectively controlled by TCR signaling.

The native LAT protein has been reported to exhibit a similar preference for the L_d lipid phase as the lipid-linked LAT in our experiments (38). However, in light of the significant number of other membrane-associated and transmembrane proteins in the cellular context of the LAT signaling condensate (59), we would refrain from extrapolating these results to predict specific details of the lipid phase associated with LAT in the natural physiological setting. The

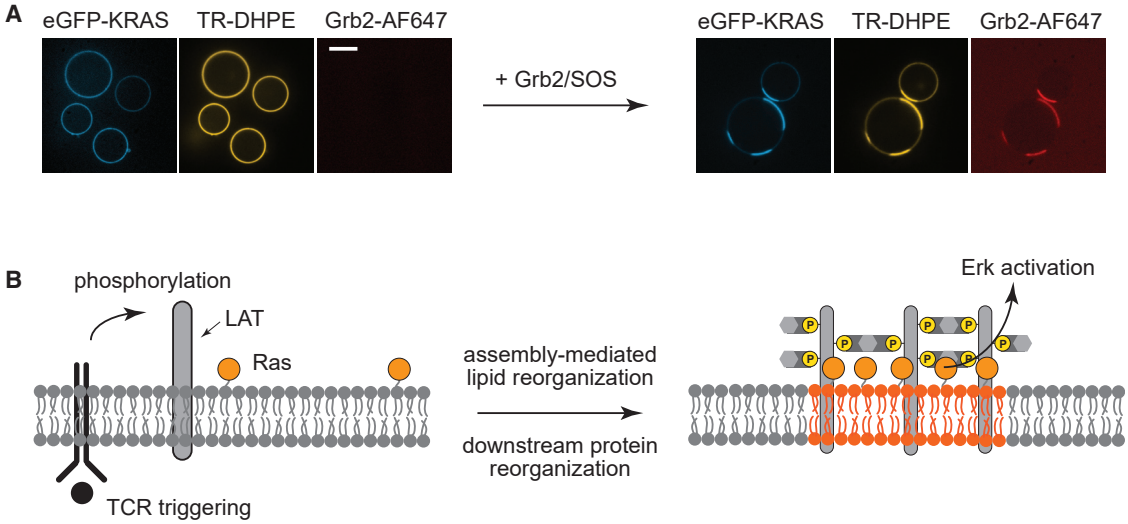

FIGURE 5 (*A*) The LAT:Grb2:SOS condensate on GUVs results in the segregation of K-Ras into the L_d region with the condensate, suggesting that spatial organization mediated by protein assemblies can propagate downstream of the signaling pathway via lipids. K-Ras templated the L_d region marked by TR on >95% of the vesicles (two independent experiments, $n \sim 30$ vesicles) with the apparent partition coefficient $K = (Ras_{in}/Ras_{out}) \sim 4.7 \pm 0.4$ (where $\pm$ denotes SEM). The temperature was 22°C, at which all of the vesicles were phase separated. Scale bars, 5 μm. (*B*) This lipid phase separation induced by protein organization may underlie lipid rafts seen in TCR clusters. To see this figure in color, go online.

important point is that LAT condensation perturbs the underlying lipids and is capable of inducing lipid phase separation now under the control of TCR signaling (Fig. 5). The LAT:Grb2:SOS protein condensate is not unique. Other two-dimensional condensates have been discovered with their own signaling specificities (50,51), and more are likely to emerge (e.g., with EGFR, which shares multivalent Grb2 and SOS interactions much like LAT). Such protein condensates on the membrane may play a broad role directly connecting receptor signaling activity with membrane lipid phase structure.

From a more physical perspective, a distinctive feature of the coupled protein-membrane system is that it exhibits phase transitions isothermally and under the control of competitive kinase-phosphatase reactions. At a single temperature and composition, the molecular interactions themselves change (as a function of LAT phosphorylation), and the phase state of the system follows. This differs from typical observations of lipid miscibility phase transitions in which the molecular properties of the lipids are fixed, and other control parameters such as temperature potentiate the phase transition (16,62,77,78). This control over LAT condensation through tyrosine phosphorylation not only enables the specific connection with cellular signaling systems, it also opens the door to various nonequilibrium chemical phenomena.

An example for such nonequilibrium phenomena can be found in a competitive lipid kinase-phosphatase reaction, which is similar to the tyrosine kinase-phosphatase competition governing LAT phosphorylation. The lipid kinase-phosphatase system has recently been observed to exhibit scale sensitivity in which the final outcome of the reaction depends on the size of the reaction system (e.g., a corralled lipid membrane in micron scales) (79). In this case, under identical concentrations of lipid kinases and phosphatases in solution, the membrane reaction system reaches a PIP_2-dominated or PIP_1-dominated (lipid kinase and phosphatase products, respectively) state based on size and degree of confinement by the corralled membranes. Even partially confined membrane features, such as filopodia, are sufficient to flip the reaction outcome, and more elaborate pattern formations occur under different geometric restrictions. As with all kinase-phosphatase competitive cycles, this example is a dissipative process that continuously consumes ATP. The system is intrinsically out of equilibrium, and the mechanism of this reaction scale sensitivity is rooted in nonequilibrium aspects of the kinetic system. The tyrosine kinase-phosphatase reactions upstream of the LAT condensate are qualitatively similar to the lipid kinase-phosphatase system mentioned above, albeit with even more complex feedback and regulatory couplings (80,81). In the case of the LAT condensate, functionally critical properties, such as nucleation threshold, size distribution, and growth-dispersion characteristics, are likely to be set by the kinase-phosphatase reactions controlling LAT phosphorylation. The LAT condensates, as well as any lipid phase structure they cause, will thus reflect the chemical states of the signaling system—including those arising from nonequilibrium processes—rather than equilibrium phase separation. At this time, very little is known about physical characteristics of LAT condensates in living cells, leaving a wealth of opportunities for detailed studies of these systems. From a functional perspective, one may speculate that lipid miscibility phase separation in living cells is inextricably coupled to numerous specific protein assemblies and signaling processes, many of which are only beginning to gain visibility.

SUPPORTING MATERIAL

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2020.09.017.

AUTHOR CONTRIBUTIONS

J.K.C., W.Y.C.H., and J.T.G. conceived the research. J.K.C., W.Y.C.H., C.B.C., and L.M.N. performed the experiments. J.K.C., W.Y.C.H., C.B.C., R.D.V., A.N.P., and J.T.G. analyzed the data and interpreted the results. J.K.C., W.Y.C.H., and J.T.G. wrote the manuscript. All authors commented on the manuscript.

ACKNOWLEDGMENTS

We thank John-Paul Denson, William K. Gillette, and Andrew G. Stephen (National Cancer Institute RAS Initiative, Frederick National Laboratory for Cancer Research) for the eGFP-K-Ras construct. We thank Young Kwang Lee, Hiu Yue Monatrice Lam, Shalini Low-Nam, and Emily C. Laubscher for assistance with the pilot experiments.

This work was supported by the Novo Nordisk Foundation Challenge Program under the Center for Geometrically Engineered Cellular Systems. Additional support was provided by National Institutes of Health, National Cancer Institute, Physical Sciences in Oncology Network project 1-U01CA202241 and by National Institutes of Health grant P01 A1091580.

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