Cytoskeletal Modulation of Lipid Interactions Regulates Lck Kinase Activity

Gurunadh R. Chichili, Robert C. Cail, and William Rodgers

From the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, and the Departments of Microbiology and Immunology and Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

Background: Rafts are important for phosphoregulation of Lck, but how they are formed and maintained in cell membranes is poorly understood.

Results: Disrupting the actomyosin cytoskeleton declusters raft lipophilic reporters and deregulates Lck.

Conclusion: The actomyosin cytoskeleton maintains lipid interactions that sustain rafts for Lck regulation.

Significance: These data provide new information regarding how rafts are maintained for Lck regulation.

The actin cytoskeleton promotes clustering of proteins associated with cholesterol-dependent rafts, but its effect on lipid interactions that form and maintain rafts is not understood. We addressed this question by determining the effect of disrupting the cytoskeleton on co-clustering of dihexadecyl-(C16)-anchored DiO and DiI, which co-enrich in ordered lipid environments such as rafts. Co-clustering was assayed by fluorescence resonance energy transfer (FRET) in labeled T cells, where rafts function in the phosphoregulation of the Src family kinase Lck. Our results show that probe co-clustering was sensitive to depolymerization of actin filaments with latrunculin B, inhibition of myosin II with blebbistatin, and treatment with neomycin to sequester phosphatidylinositol 4,5-bisphosphate. Cytoskeletal effects on lipid interactions were not restricted to order-prefering label because co-clustering of C16-anchored DiO with didodecyl (C12)-anchored DiI, which favors disordered lipids, was also reduced by Lat B and blebbistatin. Furthermore, conditions that disrupted probe co-clustering resulted in activation of Lck. These data show that the cytoskeleton globally modulates lipid interactions in the plasma membrane, and this property maintains rafts that function in Lck regulation.

Cell membranes are fluid structures, yet they retain discrete protein and lipid domains that functionally compartmentalize the bilayer. One example of this property is the cholesterol-dependent rafts, which function in signal transduction (1), membrane trafficking (2), and cell adhesion (3). In T cells, rafts are posited to modulate interactions between the Src family kinase (SFK) Lck and its regulators CD45 and Csk (4, 5). Factors that regulate the formation of rafts therefore impact cell viability and survival, and, in T cells, regulate host response to antigen.

Structurally, rafts are posited to consist of lipids that are ordered through interactions with cholesterol. Cholesterol-dependent lipid ordering to form discrete domains is represented in model membranes by combinations of cholesterol and fluid phase lipids, notably sphingolipids, that form a cholesterol-dependent liquid-ordered (Lo) phase (6–8). Some bilayer compositions contain coexisting Lα and liquid-disordered (Ld) phases (8, 9), and this is suggested to be representative of coexisting raft and nonraft environments in cell membranes (10). Coexisting ordered and disordered phases can be produced in blebs that are generated by chemical treatment of cells (11), showing that the plasma membrane contains lipid mixtures that will undergo phase separations in certain conditions.

Consistent with the membrane raft model, proteins and lipids that favor Lα lipid environments exhibit a cholesterol-dependent clustering in the plasma membrane (12, 13). Furthermore, clustering of raft markers in the plasma membrane often correlate with the F-actin content of the cell (14, 15), suggesting that the cytoskeleton can promote, by poorly understood mechanisms, lipid interactions that transition them to a more ordered state (16). This interpretation is supported by data showing that lipids in model membranes undergo ordering as a result of attachment of actin filaments to the bilayer (17). However, cytoskeletal ordering of lipids in minimalist systems such as lipid vesicles may not be representative of cell membranes. Emission from the lipophilic probe Laurdan, which is sensitive to solvent polarity, is shifted by disrupting the cell cytoskeleton in a manner that is consistent with disordering of plasma membrane lipids (16), yet this may reflect changes in probe fluorescence by parameters unrelated to lipid ordering. Also unknown is whether the effect of the cytoskeleton on lipid interactions is specific to lipids that favor ordered lipid environments such as rafts.

Most rafts have a diameter less than ~20 nm (18), thus challenging their characterization in intact cells because they are too small to visually resolve by light microscopy. Nonetheless, separate approaches exist for resolving nanoscale complexes, one being fluorescence resonance energy transfer (FRET) (13, 19–21). Measuring FRET between dialkyl forms of the carbocyanine dyes DiO and DiI, Baird and co-workers (21) showed evidence of cholesterol-dependent lipid heterogeneities in the
plasma membrane of RBL cells. However, the role of the cytoskeleton in forming these lipid complexes was not reported.

Herein, we report experiments that measured the effect of altering the cytoskeleton on FRET between membrane-anchored forms of DiO and Dil. Our data show that conditions that disrupt either the structural or functional integrity of the cytoskeleton resulted in a decrease in probe clustering associated with lipid ordering. These conditions also altered phosphorylation of Lck on its regulatory tyrosines to increase the amount of active protein. Altogether, these data favor a model where the cytoskeleton regulates formation of lipid domains that are necessary for efficient regulation of Lck.

EXPERIMENTAL PROCEDURES

Cell Culture and Sample Preparation—Jurkat T cells (clone E6-1) were prepared for microscopy by seeding 10⁶ cells onto a poly-L-lysine-coated (Sigma) coverslip, followed by washing with RPMI containing 50 mM HEPES (pH 7.4) (RPMI-HEPES), and then adding RPMI-HEPES containing either 1% dimethyl sulfoxide (Sigma) alone, or drug diluted from a 100X stock solution in dimethyl sulfoxide. Final drug concentrations were 5 μM latrunculin B (Lat B) (Calbiochem, La Jolla, CA), 50 μM blebbistatin ((–) stereoisomer) (Calbiochem), and 5 μg/ml filipin (Cayman Chemicals, Ann Arbor, MI). Incubations with either drug or vehicle alone were for 30 min at 37 °C. Alternatively, cells were treated with 20 mM neomycin overnight at room temperature of the instrument was maintained at 37 °C, and the flow rate was maintained by a base sheath pressure of 60 psi. The temperature of the instrument was maintained at 37 °C, and the flow rate was maintained by a base sheath pressure of 60 psi ± 1 psi.

Preparation of GUVs—0.05 mg of lipid containing 0.1 mole % C₁₆-DiO and C₁₂-Dil was dried on to a indium tin oxide-coated slide (Delta Technologies, Stillwater, MN), first using a gentle stream of Ar gas, followed by incubation under vacuum for 1 h at room temperature. Giant unilamellar vesicles (GUVs) were generated by electroformation as described (23), using a sandwich composed of two indium tin oxide-coated slides and containing ~0.5 ml of 300 mM sucrose in Milli-Q water (Millipore, Billerica, MA). GUVs were grown using a sinusoidal wave current (1 V, 10 Hz) for not <1 h at 60 °C. The GUV fraction was collected and diluted with an equal volume of 150 mM NaCl in Millipore water for imaging.

Flow Cytometry—Jurkat T cells were fixed in 2% (w/v) paraformaldehyde for 20 min at 37 °C and permeabilized in 0.1% (v/v) Triton X-100 in PBS-glycine for 10 min at room temperature. Staining with Texas Red-conjugated phalloidin was done as we have described (24). Cells were analyzed on an LSR II Flow Cytometer (BD Biosciences). 10,000 events were counted from each sample. Cells were gated on Forward Scatter and Side Scatter using untreated cells, and this gate was applied to the remaining samples for measurement of Texas Red fluorescence.

Cell Imaging and Analysis—Unless otherwise noted, imaging was performed using a Zeiss LSM 510 META confocal microscope (Oklahoma Medical Research Foundation imaging core facility) equipped with a 63× water objective (NA, 1.2) and a thermo-controlled chamber for maintaining the samples at 37 °C (19). Image processing and quantitation were performed using iVision imaging software (version 4.0, BioVision Technologies, Exton, PA).

FRET was measured by detecting sensitized emission of acceptor following donor excitation. Accordingly, images were acquired in three separate channels: donor channel (DiO, 488 nm excitation/505 to 550 nm emission), acceptor channel (DiI, 543 nm excitation/560 nm < emission), and FRET channel (488 nm excitation/560 nm < emission). FRET efficiency was calculated using images collected in the FRET channel. Images of cells labeled with either C₁₆-DiO or C₁₂-Dil alone were collected in the DiO and DiI channels to determine correction factors necessary to eliminate contributions from donor and acceptor bleed-through to the FRET channel (25–27). The parameter K₄ for conversion of fluorescence ratios to FRET efficiency values (see Equation 1) (27) was determined by measuring quenching of donor fluorescence following addition of acceptor. Specifically, cells labeled with C₁₆-DiO alone were imaged in the donor channel (F₀) and then re-imaged in the donor channel following addition of an equal amount of C₁₆-DiO (F₂DA). The decrease in donor fluorescence by labeling with acceptor represents the FRET efficiency, E, calculated using the equation:

$$E_{\text{quenching}} = 1 - \frac{F_{\text{DA}}}{F_{\phi}} \quad \text{(Eq. 1)}$$

K₄ was calculated from $E_{\text{quenching}}$ as described (see Equation 5 in Ref. 27).

RESULTS

Cell Labeling and Detection of FRET between Lipophilic Carboxyamine Dyes—We used dihexadecyl (C₁₆)- and didodecyl (C₁₂)-anchored forms of DiO and Dil to measure by FRET...
microscopy membrane heterogeneities associated with lipid ordering. The principal behind this approach is illustrated in Fig. 1A. Specifically, the C16 tail targets the probes to domains composed of ordered lipids, whereas C12-anchored probes favor less-ordered Ld phase lipid environments (28, 29). Accordingly, rafts composed of ordered lipids co-label with C16-anchored DiO (C16-DiO) and C16-DiI, minimizing their intermolecular distance to increase the FRET efficiency relative to that between C16-DiO and C12-DiI (21).

We show examples of the distinct affinities of the C16- and C12-anchored probes for L0 phase lipids in Fig. 1B. Each row consists of images of a GUV double-labeled with C16-DiO and C12-DiI. In the top row is a GUV containing co-existing Lo and Ld phases; C16-DiO and C12-DiI segregate due to enrichment in the separate lipid phases. Conversely, in the bottom row is a GUV containing only Ld phase lipids, and C16-DiO and C12-DiI colocalize throughout the vesicle. Measurement of multiple (n = 31) mixed L0/Ld phase GUVs showed that 20% of the C16-DiO signal colocalized with regions of C12-DiI enrichment. Thus, 80% of the C16-DiO was restricted to the Lo phase.

We controlled for probe co-clustering absent lipid ordering by measuring FRET in GUVs composed of Ld phase lipids. Specifically, demixing of probes due to unexpected interactions between C16-DiO and either C16-DiI or C12-DiI will show as an elevated FRET efficiency, which occurs following cross-linking the T cell receptor (4, 30). The cells were double-labeled using conditions that minimized internalization of the probes (see “Experimental Procedures”), while also producing efficient labeling of the plasma membrane and a FRET signal that was specific to cells that contained both DiO and DiI (Fig. 1D). To control for perturbation of the plasma membrane by the labeling, we measured the increase in intracellular Ca2+, or Ca2+ flux, which occurs following cross-linking the T cell receptor as this is sensitive to changes in plasma membrane permeability (31).

**Lipid Ordering in T Cell Plasma Membrane by Cytoskeleton**—We measured the plasma membrane of Jurkat T cells, where rafts are critical for cell regulation and stimulation through the T cell receptor (4, 30). The cells were double-labeled using conditions that minimized internalization of the probes (see “Experimental Procedures”). We observed that double-labeling cells with C16-DiO and C16-DiI had no affect on either the magnitude or duration of the Ca2+ flux (Fig. 1E), thus indicating no significant perturbation of the outer membrane by the probes.

**FIGURE 2.** C16-anchored lipophilic probes exhibit elevated FRET that is sensitive to disruption of the actomyosin cytoskeleton. A, FRET efficiency values measured in Jurkat cells double-labeled with the indicated donor-acceptor pair. Measurements were restricted to cells containing a relative fluorescence intensity of acceptor of 1500 (> 150), and a donor-to-acceptor (D:A) ratio, which represent the relative intensity of donor (D) and acceptor (A), of 1:1, 1:2, or 1:3. *, p < 0.05 by Dunnett’s multiple comparison test. For C16/C12 FRET efficiency values, 0.05 < p for both the 1:2 and 1:3 samples relative to the 1:1 sample. B, FRET efficiency values measured for C16-DiO and C12-DiI in untreated control cells and cells that were treated with filipin, Lat B, or blebbistatin (Blebb). The cells contained the indicated D:A ratio. The acceptor intensity was similar to that in A. Indicated in A and B are the average FRET efficiency for each sample (horizontal bars) and the S.E. (error bars). *, p < 0.05 by two-tailed Student’s t test; NS, 0.05 < p. Each data set passed the Bartlett’s test for equal variance (ns, 0.05 < p).
Summarized in Fig. 2A are results from measuring FRET between C16-DiO and either C16-DiI or C12-DiI in double-labeled Jurkat cells containing the indicated ratios of donor-to-acceptor (D:A). These data show co-clustering of C16-DiO and C16-DiI was significantly greater than that of C16-DiO and C12-DiI. For example, FRET efficiency for C16-anchored DiO and DiI was ~2-fold or greater than that of C16-DiO and C12-DiI at each D:A ratio. Furthermore, FRET between C16-anchored DiO and DiI was sensitive to the D:A ratio, which is another signature of co-clustering of the donor and acceptor (32). The elevated co-clustering of C16-DiO and C16-DiI was cholesterol-dependent because sequestering cholesterol with filipin decreased their FRET efficiency to values similar to that of C16-DiO and C12-DiI, and the FRET became independent of the D:A ratio (Fig. 2B). Importantly, the decrease in FRET efficiency for the C16-anchored probes was not due to anomalous quenching of the DiI by filipin because fluorescence intensities of cells labeled with C16-DiI were unchanged due to anomalous quenching of the DiI by filipin because fluorescence intensities of cells labeled with C16-DiI were unchanged upon addition of the drug (supplemental Fig. S1). Altogether, these data are consistent with the notion of cholesterol-dependent rafts in the plasma membrane that are composed of Lα phase lipids.

To determine whether co-clustering of C16-anchored DiO and DiI was sensitive to disruption of the actomyosin cytoskeleton, we measured cells treated with either latrunculin B (Lat B) to disrupt actin filaments, or blebbistatin to inhibit nonmuscle myosin II (NM II). Each of these conditions was as efficient as filipin in reducing FRET between C16-DiO and C16-DiI, and each resulted in the FRET efficiency becoming independent of the D:A ratio (Fig. 2B). Importantly, the decrease in probe co-clustering by filipin and blebbistatin was not due to a loss of F-actin, as treated and untreated cells showed similar amounts of staining by phalloidin (Fig. 3). Altogether, these data show that ordered lipid domains detected by FRET between C16-anchored probes are sensitive to conditions that either disrupt F-actin or inhibit NM II activity.

Cytoskeleton Elevates Interactions between Lα- and Lα prefering Probes—In contrast to our results with C16-DiO and C16-DiI, FRET between C16-DiO and C12-DiI was not affected by filipin, Lat B, or blebbistatin (supplemental Fig. S2). This suggests that co-clustering of C16-DiO and C12-DiI is minimal and therefore not further reduced by the respective treatments. Alternatively, lipid heterogeneities co-labeled with C16-DiO and C12-DiI may exist but were not distinguished by the FRET analysis in Fig. 2. To discriminate these separate interpretations, we employed an alternative approach to assess probe co-clustering using FRET efficiency values. This consisted of measuring the effect of acceptor concentration (F) on FRET efficiency, which can distinguish clustering events not resolved by measuring FRET over a narrow range of acceptor concentrations (32). Co-clustering of each donor-acceptor pair was quantitated as described (13), which consisted of fitting FRET efficiency (E) values to the isothermal binding equation.

\[
E = \frac{E_{\text{max}} F}{(F + K)}
\]  
(Eq. 2)

where \(K\) in Equation 2 is analogous to a disassociation constant for the donor and acceptor (13), decreasing in value as their co-clustering increases.

**FIGURE 3. Neomycin, neomycin co-treatment with filipin, and blebbistatin do not affect the F-actin content in Jurkat cells.** A, cells were stained with Texas Red-labeled phalloidin (Phalloidin-Texas Red) following the respective treatments, and the labeling was measured by flow cytometry (as described under “Experimental Procedures”). Cells treated with Lat B served as a positive control to show detection of depletion of F-actin. An unstained sample (Unstained) was measured to control for nonspecific fluorescence. The y axis (% of max) represents normalized values 1602 of cell number. The x axis (% of max) represents normalized values 1602 of cell number. Error bars represent S.D. For the neomycin-, neomycin + filipin-, blebbistatin-, and filipin-treated samples, 0.05 \(p\) relative to the untreated cells by Dunnett’s multiple comparison test. B, averaged values of staining intensity measured by flow cytometry. The data represent histogram peak intensity values were averaged from three separate trials. Error bars represent S.D. For the neomycin-, neomycin + filipin-, blebbistatin-, and filipin-treated samples, 0.05 \(p\) relative to the untreated cells by Dunnett’s multiple comparison test. p < 0.05 for all samples relative to the Lat B-treated sample. Rel. Fluor Inten., relative fluorescence intensity.

Fitted curves generated from FRET between C16-DiO and either C16-DiI or C12-DiI are shown in Fig. 4A, and \(K\) determined for each experiment is plotted in Fig. 4B. Variance in the E values that is inherent in this approach was addressed by using large data sets for the curve fitting, which minimized the 95% confidence intervals in the fitted curve (blue dashed lines). Consistent with our findings in Fig. 2 showing an elevated co-clustering of C16-anchored probes, \(K\) from FRET between C16-DiO and C16-DiI was 5-fold less than \(K\) for C16-DiO and C12-DiI. To test the specificity of the curve fitting, FRET efficiency values measured for C16-DiO and C12-DiI were fit to Equation 2 using \(K\) determined for C16-DiO to C16-DiI FRET. This resulted in a fitted curve that deviated from the experimental values and produced large and nonrandom residuals (supplemental Fig. S3), thus showing that resolved \(K\) values were specific to the respective data sets. In summary, the separate FRET donor-acceptor pairs produced distinct sets of FRET efficiency values over a range of an acceptor values, and this resulted in unique values for \(K\).
Lipid Ordering and Lck Regulation by Actomyosin Cytoskeleton

Also plotted in Fig. 4B are K values determined for cells treated with filipin, Lat B, or blebbistatin, each resolved from FRET between C₁₆-DiO and either C₁₆-DiI or C₁₂-DiI. Summarizing our results, filipin increased K for the C₁₆-DiO and C₁₆-DiI FRET pair alone, whereas K trended toward even larger values for both sets of FRET pairs when cells were treated with either Lat B or blebbistatin. Furthermore, the fitted curves for the Lat B- and blebbistatin-treated samples were linear over much of the range of acceptor values, making K approximate to the largest acceptor intensity value (Fig. 4B and supplemental Fig. S4). This property is indicative of a random or nonclustered distribution of the donor and acceptor (13), showing efficient demixing of both Lₐ and Lₕ phase probes by Lat B and blebbistatin. Thus, the cytoskeleton affected co-clustering of probes independent of their affinity for ordered lipid environments, indicating that cytoskeletal modulation of lipid interactions was not restricted to the raft Lₕ phase.

Clustering of Lipophilic Probes Requires PIP₂—To identify membrane signals that activate lipid clustering, we measured FRET in cells where the lipid co-factor phosphatidylinositol 4,5-bisphosphate (PIP₂) was sequestered using neomycin (33, 34). FRET efficiency and K values determined in this experiment are plotted in Fig. 5. These data show that treatment with neomycin decreased co-clustering of C₁₆-DiO and C₁₆-DiI, evidenced by a decrease in FRET efficiency (Fig. 5A) and an increase in K (Fig. 5B and supplemental Fig. S5). Furthermore, this effect was specific because FRET efficiency (Fig. S2) and K values (Fig. 5) determined for cells treated with the neomycin alone, neomycin + filipin, and neomycin + filipin + blebbistatin were not changed significantly by the neomycin. In a separate experiment, we observed that sequestering PIP₂ by overexpressing the pleckstrin homology domain of phospholipase C-δ caused a measurable and significant decrease in the generalized polarization of Laurdan (supplemental Fig. S6), which is a signature of decondensation of membrane lipids associated with disruption of rafts (35, 54). Because PIP₂ signals that structure the cytoskeleton are cholesterol-dependent (34), we also measured the effect of co-treating cells with neomycin and filipin on probe co-clustering. As we show in Fig. 5, these conditions disrupted co-clustering of C₁₆-DiO with both C₁₆-DiI and C₁₂-DiI, indicated by K values that trended toward the maximum acceptor intensity for each donor-acceptor pair (Fig. 5B and supplemental Fig. S5). Similarly, FRET efficiency for C₁₆-DiO and C₁₂-DiI were not changed significantly by the neomycin. In a separate experiment, we observed that sequestering PIP₂ by overexpressing the pleckstrin homology domain of phospholipase C-δ caused a measurable and significant decrease in the generalized polarization of Laurdan (supplemental Fig. S6), which is a signature of decondensation of membrane lipids associated with disruption of rafts (35, 54).
Lipid Ordering and Lck Regulation by Actomyosin Cytoskeleton

Affected phalloidin staining (Fig. 3), again showing that the changes in probe co-clustering were not due to a decrease in the amount of F-actin. Altogether, these data are evidence that cytoskeletal effects on lipid ordering require cholesterol-dependent PIP2 signals.

SFK Phosphoregulation by Signals That Modulate Lipid Interactions—SFKs undergo regulation through alternate phosphorylation and dephosphorylation of separate regulatory tyrosines. For Lck, this consists of phosphorylation of its regulatory C-terminal Tyr505 by Csk to down-regulate activity (36) and dephosphorylation of phospho-Tyr505 (pTyr505) by CD45 for activation (37).

Previous findings suggest membrane rafts are necessary to maintain negative regulation of Lck. For example, detergent fractionation studies show Csk co-associates with Lck in a detergent-resistant membrane fraction, which is posited to be representative of the composition of rafts (5, 38). Conversely, CD45 is excluded from detergent-resistant membranes (4). Similarly, treating T cells with either filipin or Lat B using conditions that disrupt rafts results in dephosphorylation of pTyr394 of Lck (19). To determine whether either neomycin or blebbistatin also affected Lck regulation, we measured phosphorylation of Tyr505 (pTyr505) in cells treated with neomycin alone, neomycin plus filipin, or blebbistatin alone. Whole cell lysates prepared from treated and untreated control Jurkat cells were immunoblotted with separate antibodies that recognized the Lck immunoblot is reported at the bottom of each lane.

FIGURE 6. Modulation of Lck tyrosine phosphorylation by the cytoskeleton. A, whole cell lysates from Jurkat and JCaM1.6 cells immunoblotted with antibody to the N terminus of Lck, Lck pTyr505, or Src pTyr416. Molecular weight, in thousands, is indicated on the right. B–D, measurement of Lck Tyr505 and Tyr394 phosphorylation, and total Lck, in cell lysates from either Jurkat cells (B and C) or J45.01 cells (D). Each sample received the indicated treatment immediately before cell lysis. The pTyr signal divided by that from the Lck immunoblot is reported at the bottom of each lane.

Lck, this residue is Tyr394, and we therefore measured the effect of the separate conditions on the phospho-Tyr394 (pTyr394) content of Jurkat cells. We detected the pTyr394 by immunoblotting with an antibody made to the phosphorylated form of the equivalent site in Src, Tyr416. Immunoblotting lysate from JCaM1.6 cells showed signal from the anti-pTyr416 antibody was specific to Lck expression (Fig. 6A), thus representing pTyr394. In Fig. 6, C and D, are representative immunoblots with the anti-pTyr394 antibody, again measuring lysates of cells that were treated with the indicated conditions. These data show that treatment with either neomycin alone, or neomycin plus filipin, increased the pTyr394 content of Lck by 2-fold and 30%, respectively. Conversely, blebbistatin produced only a nominal increase in pTyr394, and Lat B had no effect (Fig. 6D).

pTyr394 is a substrate for CD45 (40), and CD45 may therefore quench increases in pTyr394 that result from treatment with either Lat B or blebbistatin. We therefore also measured pTyr394 levels in Lat B- and blebbistatin-treated J45.01 cells, which are a CD45-deficient clone of Jurkat cells (41). This showed that blebbistatin caused a robust increase in the pTyr394 (Fig. 6D), whereas Lat B caused a modest decrease in pTyr394. In summary, Fig. 6 shows that conditions that disrupt lipid interactions that we detected by FRET results in activation of Lck, indicating that interactions between Lck and its regulators are modulated by lipid domains that are maintained by the cytoskeleton. The results with Lat B-treated cells, however, indicate that other, lipid-independent mechanisms are also important for regulating Lck during homeostasis.

DISCUSSION

We report here findings showing that nanoscopic lipid domains composed of ordered lipids are maintained by the actomyosin cytoskeleton, evidenced by declustering of C16-anchored DiO and DiI by either depolymerizing actin filaments with Lat B, or by inhibiting NM II activity with blebbistatin. Probe co-clustering was sensitive to sequestration of cholesterol with filipin, evidence that lipid domains detected by C16-DiO to C16-DiI FRET fall within the broad generalization of rafts, namely, lipid domains that form through interactions between cholesterol, other membrane lipids, and membrane proteins (42).

The phosphoinositide PIP2 and its PI3K produce phosphatidylinositol 3,4,5-trisphosphate are critical co-factors in actin polymerization and attachment of actin filaments to cell membranes. Furthermore, we observed that co-clustering of the C16-anchored dyes was reduced in cells treated with neomycin to sequester PIP2. Similarly, sequestering PIP2 by overexpressing pleckstrin homology domain of phospholipase C-6 produced a decondensation of plasma membrane lipids as reported by the generalized polarization of Laurdan. The augmented effect in disrupting probe clustering from co-treating cells with neomycin and filipin is consistent with findings that show cytoskeletal architecture is regulated by both PIP2 and cholesterol (34) and suggests PIP2 signals that modulate the cytoskeleton are compartmentalized to rafts. Similarly, we previously showed that selectively elevating or depleting raft pools of PIP2 by expressing separate forms of the polyphosphate 5‘-phosphatase Inp54p resulted in robust and distinct changes in cell mor-
Lipid Ordering and Lck Regulation by Actomyosin Cytoskeleton

The plasma membrane is suggested to exist at a critical point that is near the transition between separate fluid phases with different degrees of lipid ordering, such that small changes in composition, temperature, or other physical properties of the membrane favor formation of ordered lipid domains (42, 43). Furthermore, protein binding to the PIP2 headgroup imparts an ordering effect in its lipid hydrocarbon chains (44), which may, in turn, impact lipid interactions proximal to PIP2. Accordingly, we posit that interactions between the cytoskeleton and plasma membrane via PIP2 favor formation of ordered lipid domains of various sizes and compositions, producing the elevated FRET between the lipophilic probes that was reported here (Fig. 7). Proteins that bind both phosphoinositides and the cytoskeleton and are therefore predicted to be important in the cytoskeletal modulation of lipid ordering include ezrin-radixin-moesin, Rho GTPases, annexins, and filamin A (16, 45, 46). Finally, the lipid ordering by the cytoskeleton to stabilize rafts contrasts with the notion that cytoskeletal facilitation facilitates raft formation through caging or direct binding of proteins to the actin network that underlies the plasma membrane (47).

Disruption of probe co-clustering by inhibiting NM II without affecting the F-actin content suggests that mechanical properties of the cytoskeleton such as tension generated by myosin activity are important for lipid ordering effects by the cytoskeleton. It is interesting to note that actin filaments can exert considerable compressive force upon lipid bilayers, evidenced by robust changes in the shape of liposomes and lipid droplets that contain attached F-actin (48, 49). Furthermore, compression applied to membrane surfaces can order underlying fluid phase lipids (50). However, further studies are needed to show whether compression applied by a combination of crosslinked actin filaments and NM II activity contribute to lipid ordering and formation rafts in cell membranes.

Previous interpretations have stressed the similarities between Lo phase lipids in model membranes and that deduced for lipids in rafts. Namely, rafts, similar to Lo phase lipids, form by interactions between fluid lipids and cholesterol to form ordered lipid domains (9, 51). However, recent studies of membrane blebs show that the notion that rafts are represented by Lo phase lipids alone is likely an oversimplification. For example, blebs with different compositions and degrees of lipid ordering can be produced by relatively modest changes in preparation (52), and some species of blebs produce ordered domains that do not exclude Ld* markers (11). These properties suggest that the plasma membrane is composed of a continuum of domains with separate degrees of lipid ordering, each established by a combination of their respective protein and lipid composition. Similarly, we showed here a cytoskeleton-dependent co-clustering of the Ld/Ld* FRET pair C16-anchored DiO and C12-anchored Dil that was not affected by filipin. This suggests co-labeling of cholesterol-independent lipid domains that are also influenced by the cytoskeleton (Fig. 7). Alternatively, the cytoskeleton may produce a global effect that increases lipid ordering regardless of lipid phase.

Conditions that decreased co-clustering of the raft markers also decreased pTyr505 levels in Lck. Furthermore, we showed for the first time that, in some instances, conditions that disrupted rafts also caused activation of Lck as reported by an elevation of pTyr394. We interpret these findings as evidence that cytoskeletal rafts are important for maintaining signal quiescence in Lck. Detergent fractionation studies show evidence that Csk co-associates with Lck in rafts and that rafts sequester Lck from CD45. Both of these properties would underlie inhibition of raft pools of Lck, first by phosphorylation of Tyr505 by Csk and then sequestration of the resulting pTyr505 from CD45. Accordingly, disruption of rafts is predicted to elevate interactions between Lck and CD45 while decreasing its interactions with Csk. This would account for the observed decrease in Lck pTyr505 content in cells treated with Lat B, neomycin, and blebbistatin. The corresponding increase in pTyr394 that was observed in most conditions could occur by activation of Lck from release of its autoinhibition by pTyr505 dephosphorylation. Other proteins likely participate in the down-regulation of raft Lck because blebbistatin increased pTyr394 in CD45-deficient J45.01 cells. One candidate is PEP (PEST domain-enriched tyrosine phosphatase), which associates with Csk to inhibit SFK signaling in T cells (53). Interactions between Lck and PEP via Csk may therefore be disrupted by blebbistatin. Altogether, further study is necessary to identify these candidates and the role of rafts in regulating their interactions with Lck.

In summary, we measured FRET between lipophilic carboxy-cyanine dyes to assess lipid heterogeneities occurring in the plasma membrane of T cells. Our data show a cholesterol-dependent lipid ordering that is sensitive to disruption of F-actin and inhibition of either NM II or PIP2. Measuring tyrosine phosphorylation of Lck, we showed that conditions that disrupt lipid domains formed by the cytoskeleton also cause deregulation of Lck. Altogether, these data show cholesterol-dependent rafts that occur as a result of interactions between the cytoskel-
etion and plasma membrane and that contribute to Lck regulation.

Acknowledgments—We thank Dr. K. Rodgers and Dr. F. Lupu for critical reading of this article.

REFERENCES

1. Dykstra, M., Cherukuri, A., Sohn, H. W., Tseng, S. J., and Pierce, S. K. (2003) Location is everything: Lipid rafts and immune cell signaling. Annu. Rev. Immunol. 21, 457–481

2. Helms, J. B., and Zurzolo, C. (2004) Lipids as targeting signals: Lipid rafts and intracellular trafficking. Traffic 5, 247–254

3. delPozo, M. A., Alderson, N. B., Kiosses, W. B., Chiang, H. H., Anderson, R. G., and Schwartz, M. A. (2004) Integrins regulate Rac targeting by internalization of membrane domains. Science 303, 839–842

4. Rodgers, W., and Rose, J. K. (1996) Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. J. Cell Biol. 135, 1515–1523

5. Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korínek, V., Angelisová, P., Scherer, J., Shevchenko, A., Hlílgert, I., Cerný, J., Drbal, K., Kuramitsu, Y., Kornack, B., Horejsi, V., and Schraven, B. (2000) Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adapter protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J. Exp. Med. 191, 1591–1604

6. SankaraRao, M. B., and Thompson, T. E. (1990) Interaction of cholesterol with various glycerophospholipids and sphingomyelin. Biochemistry 29, 10670–10675

7. Baumgart, T., Hammond, A. T., Sengupta, P., Hess, S. T., Holowka, D. A., Baird, B. A., and Webb, W. W. (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. Proc. Natl. Acad. Sci. U.S.A. 104, 3165–3170

8. Veatch, S. L., and Keller, S. L. (2003) Separation of lipid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. Biophys. J. 85, 3074–3083

9. Dietrich, C., Bagatolli, L. A., Volovyk, Z. N., Thompson, N. L., Levi, M., Jacobson, K., and Gratton, E. (2001) Lipid rafts reconstituted in model membranes. Biophys. J. 80, 1417–1428

10. Timofeeva, S. and Toomre, D. (2000) Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. 1, 31–39

11. Kaiser, H. J., Lingwood, D., Levental, I., Sampiao, J. L., Kalvodova, L., Rajendran, L., and Simons, K. (2009) Order of lipid phases in model and plasma membranes. Proc. Natl. Acad. Sci. U.S.A. 106, 16645–16650

12. Varma, R., and Mayor, S. (1998) GPI-anchored proteins are organized in rafts and contribute to Lck and p56lck associated with glycolipid-enriched membrane domains. J. Cell Biol. 135, 1515–1523

13. Chichili, G. R., and Rodgers, W. (2007) Compartmentalization of phosphatidylinositol 4,5-bisphosphate signaling evidenced using targeted phosphatases. J. Biol. Chem. 283, 29920–29928

14. Veatch, S. L. (2007) Electro-formation and fluorescence microscopy of gigasomes in living cell membranes. Methods Mol. Biol. 398, 59–72

15. Zal, T., and Gascoigne, N. R. (2004) Photobleaching-corrected FRET efficiency imaging of live cells. Biophys. J. 86, 3923–3939

16. Tanter, P., Sohn, H. W., and Pierce, S. K. (2005) The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. Nat. Immunol. 6, 1168–1176

17. Chichili, G. R., Westmuckett, A. D., and Rodgers, W. (2010) T cell signal regulation by the actin cytoskeleton. J. Biol. Chem. 285, 14737–14746

18. Prasad, R., and Kilmartin, J. V. (1995) Selectivity of fluorescent lipid analogues for lipid domains. Biochemistry 34, 798–801

19. Hsu, M., Mukherjee, S., and Maxfield, F. R. (2001) Cholesterol depletion induces large scale domain segregation in living cell membranes. Proc. Natl. Acad. Sci. U.S.A. 98, 13072–13077

20. Hermiston, M. L., Xu, Z., and Weiss, A. (2003) CD45: A critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. 21, 107–137

21. Pizzolato, S., Giurisato, E., Tassi, M., Benedetti, A., Pozzan, T., and Viola, A. (2002) Lipid rafts and T cell receptor signaling: A critical re-evaluation. Eur. J. Immunol. 32, 3082–3091

22. Kenworthy, A. K., and Edidin, M. (1998) Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of < 100 Å using imaging fluorescence resonance energy transfer. J. Cell Biol. 142, 69–84

23. Gabev, E., Kasianowicz, J., Abbott, T., and McLaughlin, S. (1989) Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2). Biochim. Biophys. Acta 979, 105–112

24. Kwiat, J., Bose, S., Fooksman, D., Margolis, L., Sheehy, M., and Edidin, M. (2003) Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. Proc. Natl. Acad. Sci. U.S.A. 100, 13964–13969

25. Gaus, K., Gratton, E., Kable, E. P., Jones, A. S., Gelissen, I., Kriithardies, L., and Jessup, W. (2003) Visualizing lipid structure and raft domains in living cells with two-photon microscopy. Proc. Natl. Acad. Sci. U.S.A. 100, 15554–15559

26. Chow, L. M., Fournel, M., Davidson, D., and Veillette, A. (1993) Negative regulation of T-cell receptor signaling by tyrosine protein kinase p56lck. Nature 365, 156–160

27. Koretzky, G. A., Ricci, J., Schultz, T., and Weiss, A. (1991) Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. Proc. Natl. Acad. Sci. U.S.A. 88, 2037–2041

28. Kawabuchi, M., Saromi, Y., Takak, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovskiy, A., and Okada, M. (2000) Transmembrane phosphoprotein Cbp regulates the activities of Src family tyrosine kinases. Nature 404, 999–1003

29. Hardwick, J. S., and Sefton, B. M. (1995) Activation of the Lck tyrosine kinase by hydrogen peroxide requires the phosphorylation of Tyr-394. Proc. Natl. Acad. Sci. U.S.A. 92, 4527–4531

30. Baez, M., Gamble, J., Tooze, R., Higgins, D., Yang, F. T., O’Brien, P. C., Coleman, N., Pingel, S., Turner, M., and Alexander, D. R. (2000) Development of T-leukemias in CD45 tyrosine phosphatase-deficient mutant lck mice. EMBO J. 19, 4644–4654

31. Koretzky, G. A., Kohmetscher, M. A., Kadlec, T., and Weiss, A. (1992)
Lipid Ordering and Lck Regulation by Actomyosin Cytoskeleton

Restoration of T cell receptor-mediated signal transduction by transfection of CD45 cDNA into a CD45-deficient variant of the Jurkat T cell line. *J. Immunol.* **149**, 1138–1142

42. Simons, K., and Gerl, M. J. (2010) Revitalizing membrane rafts: New tools and insights. *Nat. Rev. Mol. Cell Biol.* **11**, 688–699

43. Lingwood, D., Ries, J., Schwille, P., and Simons, K. (2008) Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 10005–10010

44. Tong, J., Nguyen, L., Vidal, A., Simon, S. A., Skene, J. H., and McIntosh, T. J. (2008) Role of GAP-43 in sequestering phosphatidylinositol 4,5-bisphosphate to Raft bilayers. *Biophys. J.* **94**, 125–133

45. Stossel, T. P., Condeelis, J., Cooley, L., Hartwig, J. H., Noegel, A., Schleicher, M., and Shapiro, S. S. (2001) Filamins as integrators of cell mechanics and signaling. *Nat. Rev. Mol. Cell Biol.* **2**, 138–145

46. Gerke, V., Creutz, C. E., and Moss, S. E. (2005) Annexins: Linking Ca$^{2+}$ signaling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **6**, 449–461

47. Kusumi, A., Koyama-Honda, I., and Suzuki, K. (2004) Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic* **5**, 213–230

48. Trichet, L., Campás, O., Sykes, C., and Plastino, J. (2007) VASP governs actin dynamics by modulating filament anchoring. *Biophys. J.* **92**, 1081–1089

49. Delatour, V., Helfer, E., Didry, D., Lê, K. H., Gaucher, J. F., Carlier, M. F., and Romet-Lemonne, G. (2008) Arp2/3 controls the motile behavior of N-WASP-functionalized GUVs and modulates N-WASP surface distribution by mediating transient links with actin filaments. *Biophys. J.* **94**, 4890–4905

50. McConnell, H. M., Tamm, L. K., and Weis, R. M. (1984) Periodic structures in lipid monolayer phase transitions. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3249–3253

51. Ahmed, S. N., Brown, D. A., and London, E. (1997) On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: Physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. *Biochemistry* **36**, 10944–10953

52. Levental, I., Grgubek, M., and Simons, K. (2011) Raft domains of variable properties and compositions in plasma membrane vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 11411–11416

53. Cloutier, J. F., and Veillette, A. (1999) Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase. *J. Exp. Med.* **189**, 111–121

54. Gaus, K., Zech, T., and Harder, T. (2006) Visualizing membrane microdomains by Laurdan 2-photon microscopy. *Mol. Membr. Biol.* **23**, 41–48