Cell membranes are laterally organized into functionally discrete domains that include the cholesterol-dependent membrane “rafts.” However, how membrane domains are established and maintained remains unresolved and controversial but often requires the actin cytoskeleton. In this study, we used fluorescence resonance energy transfer to measure the role of the actin cytoskeleton in the co-clustering of membrane raft-associated fluorescent proteins (FPs) and FPs targeted to the nonraft membrane fraction. By fitting the fluorescence resonance energy transfer data to an isothermal binding equation, we observed a specific co-clustering of raft-associated donor and acceptor probes that was sensitive to latrunculin B (Lat B), which disrupts the actin cytoskeleton. Conversely, treating with jasplakinolide to enhance actin polymerization increased co-clustering of the raft-associated FPs over that of the nonraft probes. We also observed by immunoblotting experiments that the actin-dependent co-clustering coincided with regulation of the raft-associated Src family kinase Lck. Specifically, Lat B decreased the phosphorylation of the C-terminal regulatory tyrosine of Lck (Tyr605), and combining the Lat B with filipin further decreased the Tyr605 phosphorylation. Furthermore, the Lat B-dependent changes in Lck regulation required CD45 because no significant changes occurred in treated T cells lacking CD45 expression. These data define a role for the actin cytoskeleton in promoting co-clustering of raft-associated proteins and show that this property is important toward regulating raft-associated signaling proteins such as Lck.

Current models define cell membranes to be structurally heterogeneous in nature, composed of discrete domains with unique physical and biological properties. This lateral heterogeneity is essential for many membrane functions, including cell signaling (1, 2), cell motility (3), protein and lipid uptake (4, 5), and even the uptake and assembly of infectious agents (6, 7). However, studies of membrane structure and function remain challenging, because functionally discrete domains are often below the resolution of light microscopy. Accordingly, properties of cell membranes and membrane domains remain unclear and even controversial (8).

An important example of domains in biological membranes is the cholesterol-dependent membrane rafts. In the membrane raft model, rafts form through associations between cholesterol and other membrane lipids, giving rise to a discrete lipid phase with which specific membrane proteins associate (9). Many studies have characterized raft domains using membrane fractionation, where it is postulated that rafts are represented by a nonionic detergent-resistant, glycolipid-enriched membrane fraction that is present in cell lysates (10). Notably, however, some investigators contend that this fraction is an artifact of detergent lysis, and the membrane raft model is inaccurate (8). Supporting the notion that the detergent-resistant glycolipid-enriched membrane fraction is representative of membrane domains in intact cells, separate studies have demonstrated a protein and lipid clustering that is specific to glycolipid-enriched membrane-associated molecules (11–17).

Imaging studies of raft-associated probes suggest that the rafts are heterogeneous in nature (18), ranging in size from nanoclusters that are ~5 nm in diameter and containing no more than a few protein molecules (14), to larger nanodomains that are ~25–100 nm in size (12, 19, 20). Finally, fluorescence imaging experiments have demonstrated micron-size raft macrodomains that are detergent-resistant and enriched with raft-associated molecules (3, 15, 16, 21–23). One example of the macrodomains is the immunological synapse in stimulated lymphocytes (24, 25), which forms where lymphocytes contact a cell containing antigen.

In T cells and other lymphocytes, evidence of membrane rafts also comes from studies of signaling proteins in the outer membrane. For example, enriched in the membrane raft fraction are proteins that participate in signaling from the surface antigen receptors, and their targeting to the raft fraction is often necessary for efficient cell activation (26). One such enzyme is the Src family kinase Lck, which functions in signaling from the T cell receptor following binding to major histocompatibility complex-peptide complexes. Disruption of Lck targeting to rafts attenuates T cell activation (27), and in resting T cells, membrane rafts function in regulating Lck (28, 29).

The factors that govern formation of membrane domains, including membrane rafts, continue to be elucidated. One such factor is the actin cytoskeleton, which can affect the lateral distribution and mobility of membrane proteins (30, 31). In relation to rafts, actin occurs in the detergent-resistant raft fraction...
(23), and actin-filaments co-associate with known membrane raft markers (16, 23). The actin cytoskeleton is also necessary for formation of some types of raft macrodomains in lymphocytes (16, 23, 32). However, the degree to which the actin cytoskeleton participates in establishing membrane rafts, such as its contribution toward protein clustering relative to that of the cholesterol-dependent lipid ordering, is not established. Importantly, the actin cytoskeleton is a dynamic structure that changes in response to extracellular signals, and it may therefore represent one mechanism for governing the size and distribution of membrane rafts in the plasma membrane.

By measuring fluorescence resonance energy transfer (FRET)\(^2\) between separate membrane-anchored fluorescent proteins, we show here a specific clustering of raft-associated membrane probes by the actin cytoskeleton. By immunoblotting, we also show the actin cytoskeleton is necessary for effective regulation of Lck. Similar effects on protein co-clustering and Lck regulation were observed in cells where cholesterol was sequestered using filipin. We conclude that both cholesterol and the actin cytoskeleton are necessary in establishing a membrane environment that provides for efficient regulation of raft-associated enzymes such as Lck.

**EXPERIMENTAL PROCEDURES**

**Gene Construction**—Generation of CFP and YFP fusion proteins containing either the first 10 amino acids of Lck (L\(_{10}\)) or the first 15 residues of Src (S\(_{15}\)) was performed as described previously (33). The sense and antisense oligonucleotides (33) for the L\(_{10}\) and S\(_{15}\) sequences were annealed and subcloned into the Smal site of pWay20 (34) containing CFP or YFP in place of GFP. A fragment encoding the first 36 residues of linker for activation of T cells (LAT\(_{36}\)) was amplified from the full-length protein using the following primers: 5'-CATCATCTAGAAATGGAGGAGGCCATCTCTGG-3' (coding); and 5'-TCTAATGATCTTTTGTGTGTGTGTGTGTGTCG-AGGGCTGGTGC-3' (noncoding). The underlined nucleotides represent restriction sites for XbaI and EcoRI in the coding and noncoding primers, respectively. The amplified product was digested and subcloned into the XbaI and EcoRI sites of pcDNA 3.1 (Invitrogen), upstream and in-frame to YFP. Lyn-CFP-YFP and CFP-T2DN-YFP have been described previously (17) and were generously provided by Dr. S. Pierce (National Institutes of Health).

**Cell Culture and Transfection**—Jurkat T cells (clone E6-1) were maintained in medium containing RPMI 1640 supplemented with antibiotics and 10% fetal bovine serum at 37 °C in the presence of 5% CO\(_2\). For gene expression, the cells were transfected by electroporation (Gene Pulser II; Bio-Rad) as described (23). For transfection, 10\(^6\) cells were suspended in 0.5 ml of RPMI and containing 25 μg of plasmid DNA. Settings of 330 V and 960 μF were used for electroporation. 48 h post-transfection, viable cells were separated by centrifuging the samples over Cellgro\textsuperscript{TM} (MediaTech, Inc., Herndon, VA). Stable clones expressing CFP fusion proteins were selected by limiting dilution using medium containing G418 (Invitrogen) at a concentration of 1.0 mg/ml. Following drug selection, the clones were enriched for protein expression by flow cytometry and maintained in medium containing 500 μg/ml G418.

**Cell Lysis, Equilibrium Centrifugation, and Immunoblotting**—For sucrose gradient equilibrium centrifugation experiments, 10\(^7\) cells were lysed with 1.0 ml of a 1% Triton X-100 solution in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM EDTA (TNE). For lysis, the samples were incubated with the detergent solution for 20 min at 4 °C and then processed with a Dounce homogenizer (8 strokes). Intact nuclei and large cell debris were removed by centrifuging (Eppendorf model 5417C; Brinkman Instruments, Westbury, NY) at 4000 rpm at 4 °C for 5 min. The supernatant was collected and diluted with an equal volume of an 85% sucrose solution in TNE. After gently mixing, the sample was overlaid in a SW50.1 rotor tube with 3.0 ml of a 30% and 1.5 ml of a 5% solution, each in TNE. The samples were centrifuged for 16–18 h at 200,000 × g. Following centrifugation, the gradients were harvested by fractionating from the top. Approximately 10% of each fraction was separated by gel electrophoresis. The fluorescent probes were detected by immunoblotting using a monoclonal antibody specific to GFP that recognizes YFP and CFP (Covance Research Products, Richmond, CA).

Assays of Lck regulation began by lysing 10\(^6\) cells in 100 μl of Laemmli sample buffer containing 1% 2-mercaptoethanol, followed by incubation at 100 °C for 5 min. After lysis, equivalent amounts of the samples were separated by gel electrophoresis and immunoblotted using monoclonal antibody to Lck (clone 2B; BD Biosciences, San Jose, CA) or rabbit antibody to Tyr(P)\textsuperscript{605} of Lck (Cell Signaling Technology; Danvers, MA). All of the immunoblots were developed by ECL (Amersham Biosciences) and detected using a Lumilager work station (Roche Applied Science).

**Fluorescence Microscopy and Image Analysis**—The samples were prepared for microscopy by seeding ~10\(^6\) cells onto a poly-L-lysine (Sigma)-coated coverslip and then fixed by incubating in a 2% solution of paraformaldehyde for 30 min at room temperature. For the drug treatments, seeded coverslips were washed with RPMI containing 50 mM HEPES (pH 7.4) (RPMI-HEPES). Next, the medium was replaced with RPMI-HEPES containing either 1% Me\(_2\)SO (Sigma) alone, or drug diluted from a 100× stock solution in Me\(_2\)SO. Final drug concentrations were 5 μM latrunculin B (Lat B) (Calbiochem, La Jolla, CA), 0.5 μM jasplakinolide (Invitrogen), and 5 μg/ml filipin (Cayman Chemicals, Ann Arbor, MI). All of the incubations were for 30 min at 37 °C.

Microscopy was performed using a Zeiss LSM 510 META confocal microscope. CFP was excited at 458 nm, and emission was detected at 473 and 505 nm. YFP was excited at 514 nm, and emission was detected from 530 to 600 nm. The images were collected using a 63× oil objective (NA 1.2), and recorded in 12-bit mode at a scan rate of 2.56 μs/pixel. All of the image processing and quantitation was performed using IPLab (BD Biosciences).

FRET was measured based on the increase in the CFP signal following photobleaching of the YFP. Photobleaching of YFP

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\(^2\)The abbreviations used are: FRET, fluorescence resonance energy transfer; FP, fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; LAT, linker for activation in T cells; Lat B, latrunculin B; TXS, Triton X-100 soluble; PIP\(_2\), phosphatidylinositol 4,5-biphosphate.
FRET Detection of an Actin-dependent Clustering of Raft Proteins

FIGURE 1. Membrane targeting of fluorescent proteins by the \( L_{10} \), \( S_{15} \), and \( LAT_{36} \) sequences. A, the FPs CFP and YFP were targeted to either the raft or the nonraft membrane fraction, using minimal membrane-anchoring signals. \( L_{10} \) and \( S_{15} \) represent the N-terminal 10 and 15 amino acids of Lck and Src, respectively. \( LAT_{36} \) is the first 36 amino acids of LAT and contains the extracellular and transmembrane domains of full-length protein, plus the membrane-proximal cysteines that are palmitoylated. The sites of myristoylation (Myr) and palmitoylation (Palm) for each of the constructs are indicated. β, membrane fractionation experiments showing the relative association of the \( L_{10}, LAT_{36}, \) and \( S_{15} \)-anchored proteins with the detergent-resistant raft fraction. The gradients were performed as described under "Experimental Procedures" using transfected Jurkat T cells expressing either GFP (top and middle) or CFP (bottom) fusion proteins. Fraction 1 represents the top of the gradient, and the raft and TXS membrane fractions occurred in the indicated gradient fractions. Note the doublet for the \( LAT_{36}-YFP \) in the TXS fraction, which may be due to protein modification, such as phosphorylation of Ser36 of the \( LAT_{36} \) domain. Molecular weights are in the thousands, and the relative amount of each protein associated with the raft fraction is indicated. The confocal images (right) are of Jurkat cells expressing the respective membrane anchors fused to YFP. The white bar represents 5 \( \mu \)m.

was restricted to a region of interest \( 8 \times 5 \) pixels (2.1 \( \times \) 1.3 \( \mu \)m) in size and was performed by illuminating the sample for 750 iterations (~45 s) at 514 nm with the laser at full power. Four images were acquired for each region of interest: 1) an image of YFP fluorescence before bleach (YFP\_pre), 2) an image of CFP fluorescence before bleach (CFP\_pre), 3) an image of CFP fluorescence after bleach (CFP\_post), and 4) an image of CFP fluorescence after bleach (CFP\_post). In addition, to correct for bleaching of CFP during YFP bleaching (CFP\_YFP bleach), cells expressing CFP alone were taken through all the steps of FRET measurement. Cells expressing only YFP were used to determine the YFP bleed-through into the CFP channel (CFP\_YFP). Fluorescence intensities of each region of interest were measured on a pixel-by-pixel basis, and measurement of YFP\_pre and YFP\_post showed the photobleaching extinguished >95% of the YFP fluorescence.

CFP\_pre was corrected for YFP bleed-through into the CFP channel using the following operation.

\[
\text{CFP}_\text{pre, corrected} = \text{CFP}_\text{pre} - \text{CFP}_\text{YFP} \tag{Eq. 1}
\]

CFP\_post bleach was corrected for both bleaching of CFP during YFP bleaching and YFP bleed-through by the following equation.

\[
\text{CFP}_\text{post, corrected} = (\text{CFP}_\text{post} - \text{CFP}_\text{YFP}) + \text{CFP}_\text{YFP bleach} \tag{Eq. 2}
\]

From the corrected values, FRET efficiency (E%) was calculated as follows.

\[
E\% = \left( \frac{\text{CFP}_\text{post, corrected} - \text{CFP}_\text{pre, corrected}}{\text{CFP}_\text{post, corrected}} \right) \times 100 \tag{Eq. 3}
\]

FRET was measured in 75–100 separate cells, restricted to those cells where the expression of the donor and acceptor were approximately equal. Co-clustering was quantitated by fitting plots of E% versus acceptor intensity to Equation 4 (below). Curve fitting and all of the statistical analysis were performed using Igor Pro (WaveMetrics, Lake Oswego, OR). Standard error was determined as described (35) using standard deviations from the curve fitting. The statistical significance of the differences in K values was determined using a two-tailed Student’s t test for distributions of unequal variance (Equations 14.2.3 and 14.2.4 in Ref. 36). The K values determined to be significantly different are indicated by asterisks, which represent a probability greater than 99.99% that the two values are different.

RESULTS

Targeting of Fluorescent Proteins to the Raft and Nonraft Membrane Fractions—The membrane-targeted FPs used in this study are illustrated in Fig. 1A. Specifically, CFP and YFP served as the FRET donor and acceptor, respectively. Each was targeted to the membrane fraction of Jurkat T cells by either the N-terminal 10 residues of Lck (\( L_{10} \)), the N-terminal 15 residues of Src (\( S_{15} \)), or the N-terminal 36 residues of linker for activation of T cells (\( LAT_{36} \)). We have described the \( L_{10} \) and \( S_{15} \) membrane targeting signals previously (33), where it was shown the \( L_{10} \) sequence targets FPs to the detergent-resistant membrane raft fraction and the \( S_{15} \) signal limits FPs to the Triton X-100-soluble (TXS) nonraft membrane fraction. \( LAT_{36} \) contains the transmembrane domain of LAT but lacks its cytoplasmic domain with associated linker functions (37). Furthermore, the \( LAT_{36} \) sequence targets fusion proteins to the raft fraction (38) because of palmitoylation of a set of membrane-proximal cysteine residues (39).
images showing each membrane anchor efficiently targeted the FPs to the plasma membrane.

Visualization of FRET by Acceptor Photobleaching—To establish detection of FRET, we first measured energy transfer in T cells expressing a control peptide consisting of CFP and YFP separated by a two-amino acid linker and containing the membrane-anchoring signal of Lyn (Lyn-CFP-YFP). The energy transfer efficiency ($E_\%$) was measured based on the increase in donor fluorescence following photobleaching of the acceptor (40). Furthermore, to show that observed changes in CFP fluorescence following YFP photobleaching were specific to FRET, measurements were made using cells expressing a second control peptide containing CFP and YFP separated by a 236-amino acid linker (CFP-T2DN-YFP).

Examples of FRET measurements of the CFP-YFP fusion peptides are shown in Fig. 2A. The yellow box in each image represents the region where the acceptor was photobleached. Accompanying the images are plots of the membrane fluorescence intensity in each channel before (red) and after (green) photobleaching. In the graphs, the y axis represents relative fluorescence intensity of the plasma membrane, and the x axis corresponds to the distance around the periphery of the cell. The vertical lines indicate the boundaries of the region that was photobleached. Note the increase in CFP fluorescence following photobleaching of the YFP specific to the Lyn-CFP-YFP molecule.

FIGURE 2. Detection of FRET by acceptor photobleaching. A, FRET measurements of Jurkat T cells expressing fusion proteins containing CFP and YFP. Lyn-CFP-YFP is membrane-anchored protein and the CFP and YFP are separated by only two amino acids. The CFP-T2DN-YFP is a soluble protein that contains a 236-amino acid linker between the CFP and the YFP. For each, FRET was detected by measuring for an increase in CFP fluorescence following photobleaching of the YFP. The images were collected in the CFP (top) and YFP (bottom) channels before (left) and after (right) photobleaching of the acceptor. The yellow square in each image marks the region that was photobleached in the YFP channel, and an enlarged view of the region is shown in the inset. Accompanying the images are plots of the membrane fluorescence intensity in each channel before (red) and after (green) photobleaching. In the graphs, the y axis represents relative fluorescence intensity of the plasma membrane, and the x axis corresponds to the distance around the periphery of the cell. The vertical lines indicate the boundaries of the region that was photobleached. Note the increase in CFP fluorescence following photobleaching of the YFP specific to the Lyn-CFP-YFP molecule.

The white bar represents 5 μm. B, theoretical predictions of the effect of random (left) and clustered (right) distributions of donor and acceptor molecules on FRET efficiency ($E_\%$). If random, then $E_\%$ increases with acceptor concentration. However, a clustered distribution results in $E_\%$ becoming saturated because of confinement of the donor and acceptor molecules to membrane domains. The illustration was adapted from Ref. 13. C and D, fitted curves from FRET experiments measured in cells expressing either Lyn-CFP-YFP (solid squares) or CFP-T2DN-YFP (open squares) (C) or between L10-CFP and L10-YFP (D) in cells treated with methyl-β-cyclodextrin and sphingomyelinase (SMase). $E_\%$ was determined over a range of acceptor intensities, and the values are plotted versus the prebleach acceptor intensity. In D, the donor-acceptor ratio was 1:1 for each cell measured. The red lines represent curves generated by fitting the $E_\%$ values to Equation 4, and the blue dashed lines represent the 95% confidence interval for each fitted curve. Also shown are the residual differences between measured and predicted $E_\%$ values for each fitted curve. The values determined for $K \pm$ S.E. are 12 ± 3 (Lyn-CFP-YFP), 1710 ± 230 (CFP-T2DN-YFP), and 69 ± 9 (L10-CFP/L10-YFP, + methyl-β-cyclodextrin and sphingomyelinase).
this property is illustrated in Fig. 2B. First, in the absence of
co-clustering, \( E\% \) increases linearly with increasing acceptor as
the distance between the donor and acceptor decreases propor-
tionally because of molecular crowding. In contrast, when the
donor and acceptor co-cluster, such as through co-association
with membrane domains, the distance between the donor-ac-
ceptor pair reaches a minimal distance, after which
\( E\% \) no lon-\nger increases with increasing acceptor concentration. Tsien
and co-workers (13) showed the associative properties of the
donor and acceptor pair such as that illustrated in Fig. 2
can be
determined by the parameter \( K \) in the following isothermal
binding equation,

\[
E\% = \frac{E_{\text{max}}F}{(F + K)} \quad \text{(Eq. 4)}
\]

where \( F \) represents the fluorescence intensity of the acceptor,
which we defined as the prebleach intensity of the YFP fluo-
res-
cence. Based on Equation 4, \( K \) is analogous to a dissociation constant of the donor and acceptor pair, approaching zero as the degree of
coclustering of the fluorescent pro-
teins increases.

In Fig. 2C are examples of \( K \) re-
representing the relative co-clustering of donor and acceptor pairs
using FRET data from the Lyn-
CFP-YFP and CFP-T2DN-YFP fusion proteins. In the experiment
with Lyn-CPF-YFP, \( K \) approached zero (\( K = 12 \)), reflecting an appar-
ent co-clustering that is due to an
adjacent donor and acceptor in the
fusion protein. In contrast, for CFP-
T2DN-YFP, where we predicted the
apparent co-clustering to be mini-
mal because of a large separation of
the donor and acceptor pair,
\( K \) is 2
orders of magnitude larger (\( K =
1710 \)) than that of the Lyn-CPF-
YFP. Note also for each fit that the
residual differences between the
experimental and predicted values
for \( E\% \) are minimal and distributed
randomly about zero, thus indicat-
ing a favorable fit of the experimen-
tal data to Equation 4. In contrast,
fitting the FRET data from Lyn-
CFP-YFP to Equation 4 but con-
strained by the \( K \) determined for
CFP-T2DN-YFP resulted in a large
deviation of the residual differences
from zero (supplemental Fig. S1,
top
row). Thus, application of Equation
4 reliably discriminates the co-clus-
tering of the separate donor-accep-
tor pairs.

As an additional control, we
determined the effect of protein aggregation on the apparent
coclustering of the L10-anchored probes (Fig. 2C). Specifically,
we measured the FRET between L10-CFP and L10-YFP in cells
treated with methyl-/H9252-
cyclodextrin and sphingomyelinase,
which represent conditions where raft-associated proteins
aggregate (43, 44). \( K \) for the co-treated samples approached
that of the Lyn-CFP-YFP (\( K =
68 \)), and this value is approxi-
mately one-fifth that determined for L10-CFP and L10-YFP in
untreated cells (Fig. 3). These data therefore further validate the
approach of using \( K \) to distinguish the relative co-clustering of
separate membrane proteins.

Cholesterol-dependent Co-clustering of Membrane-anchored
Fluorescent Proteins—To determine the relative co-clustering
of the membrane-anchored FPs, we fitted FRET data to Equa-
tion 4 from cells co-expressing L10- or S 15-CFP with L10-
LAT36-, or S15-YFP (Fig. 3; see also Fig. 5). Consistent with
the membrane raft model, these data show a specific and cholesterol-
dependent co-clustering of probes that associate with the detergent-resistant raft fraction. For example, the lowest values for \( K \) occurred in samples double-labeled with \( L_{10}^-\)CFP and either \( L_{10}^-\)YFP or \( \text{LAT}_{36}^-\)YFP (\( K = 263 \) and 154, respectively), and these values were up to 11-fold lower than \( K \) determined for cells co-expressing an \( S_{15}^-\)anchored probe with the \( L_{10}^-\) or \( \text{LAT}_{36}^-\)-FPs (1100 < \( K \) < 1700) (Fig. 3). Furthermore, cells co-expressing \( L_{10}^-\)CFP with either \( L_{10}^-\) or \( \text{LAT}_{36}^-\)-YFP and treated with filipin to sequester cholesterol (45) and disrupt the membrane raft fraction (Fig. 4) exhibited up to a 10-fold increase in \( K \). However, disruption of protein co-clustering by filipin was specific to donor and acceptor pairs where both associated with the raft fraction, because cells co-expressing \( L_{10}^-\)CFP and \( S_{15}^-\)YFP exhibited a decrease in \( K \) in these conditions (Fig. 5, A and D). The observed increase in co-clustering of \( L_{10}^-\)CFP and \( S_{15}^-\)-YFP in the filipin-treated samples may reflect loss of a cholesterol-dependent segregation of these probes. Also note that \( K \) determined in cells co-expressing \( S_{15}^-\)CFP and \( S_{15}^-\)-YFP approached that for samples double-labeled with the raft-associated probes (Fig. 3), thus indicating measurable co-clustering of the \( S_{15}^-\)-anchored proteins in non-raft pools of the membrane.

As described for the control fusion proteins, we also tested the specificity of the observed curve fitting by performing a forced fit using experimental data from samples double-labeled with \( L_{10}^-\)CFP and \( L_{10}^-\)YFP. Fitting these data to Equation 4 using \( K \) from cells co-expressing \( L_{10}^-\)CFP and \( S_{15}^-\)-YFP resulted in a significant increase in the residual differences, and their deviation from a distribution centered at zero (supplemental Fig. S1, middle row). Thus, the separate \( K \) values between the samples double-labeled with \( L_{10}^-\)CFP and \( L_{10}^-\)YFP and those co-expressing \( L_{10}^-\)CFP with \( S_{15}^-\)-YFP reflect authentic differences in the curve fitting. Similarly, a Student’s t test ("Experimental Procedures") showed that the \( K \) values for these samples are significantly different (asterisk in Fig. 3B).

Co-clustering of Raft-associated Probes Is Actin-dependent— Micron-size raft domains, such as the immunological synapse in stimulated T cells, associate with actin filaments (16). Similarly, the actin cytoskeleton is necessary for formation of large membrane raft complexes (32). Because the associative properties of the actin cytoskeleton and membrane rafts may reflect an actin-dependent clustering of raft proteins, we asked whether co-clustering of the \( L_{10}^-\)CFP with other membrane-anchored FPs was actin-dependent. Accordingly, we treated cells co-expressing \( L_{10}^-\)CFP and either \( L_{10}^-\), \( \text{LAT}_{36}^-\), or \( S_{15}^-\)-YFP with Lat B to disrupt the actin cytoskeleton. Interestingly, although the Lat B had no effect on association of the \( L_{10}^-\)-anchored probes with the membrane raft fraction (Fig. 4), it did disrupt co-clustering of \( L_{10}^-\)CFP with \( L_{10}^-\) and \( \text{LAT}_{36}^-\)YFP (Fig. 5). For example, \( K \) for the Lat B-treated samples co-expressing \( L_{10}^-\)CFP and \( L_{10}^-\)YFP increased 6-fold over that of untreated cells (\( K = 1562 \) versus 263) (Fig. 5, A and B), and a similar relative increase in \( K \) occurred in samples double-labeled with \( L_{10}^-\)CFP and \( \text{LAT}_{36}^-\)YFP (\( K = 955 \) versus 154) (Fig. 5, A and C). In contrast, co-clustering of the \( L_{10}^-\)CFP and \( S_{15}^-\)-YFP increased modestly in these conditions, because \( K \) decreased from 1709 for the

**FRET Detection of an Actin-dependent Clustering of Raft Proteins**

**FIGURE 4.** Membrane fractionation of Lat B- and filipin-treated T cells. The raft and TXS membrane fractions of Jurkat T cells \( L_{10}^-\)GFP were separated by sucrose gradient equilibrium centrifugation of Triton X-100 cell lysates. Prior to lysis, the samples were either untreated (top) or pretreated with either Lat B (middle) or filipin (bottom). The molecular weight is in thousands, and the fraction of \( L_{10}^-\)GFP protein in the raft fraction of each sample is indicated. The confocal images (right) are of \( L_{10}^-\)YFP in Jurkat cells in the respective conditions. The white bars represent 5 \( \mu m \).

**FIGURE 5.** Specific co-clustering of raft-associated probes by the actin cytoskeleton and membrane cholesterol. A, graphs showing fitted curves and residual differences from measurement of FRET between \( L_{10}^-\)CFP and either \( L_{10}^-\)YFP (left), \( S_{15}^-\)YFP (middle), or \( \text{LAT}_{36}^-\)YFP (right). The samples were pretreated with Lat B (top, filipin (middle), or both Lat B and filipin (bottom). B–D, values determined for \( K \) ± S.E. from the fitted curves in A. For FRET between \( L_{10}^-\)CFP and \( L_{10}^-\)YFP, \( K \) ± S.E. = 1562 ± 91, 887 ± 47, and 2169 ± 141 for the Lat B-, filipin-, and co-treated samples, respectively. For samples double-labeled with \( L_{10}^-\)CFP and \( \text{LAT}_{36}^-\)YFP, \( K \) ± S.E. = 955 ± 72, 1477 ± 115 and 2234 ± 108 for sample treated with Lat B-, filipin-, and co-treated samples, respectively. The samples expressing \( L_{10}^-\)CFP and \( S_{15}^-\)-YFP had values for \( K \) ± S.E. = 1247 ± 66, 908 ± 33, and 1698 ± 139 for Lat B-, filipin-, and co-treated samples, respectively. The values for \( K \) for the untreated samples in B–D are the same as that presented in Fig. 3. All of the asterisks indicate a probability of >99.99% that the paired \( K \) values are different. The black asterisks represent a comparison with untreated control. The colored asterisk is a comparison of the filipin- and Lat B-treated samples. ns represents not significantly different from the control.
untreated samples to 1247 for those that received the Lat B (Fig. 5, A and D). Thus, the disruption of protein co-clustering by Lat B was again specific to where both the donor and acceptor were raft-associated.

Interestingly, comparing the results from treatments with either filipin or Lat B in Fig. 5 shows that in some cases the actin cytoskeleton was more significant than cholesterol toward promoting co-clustering of the raft-associated probes. For example, in the cells double-labeled with L10-CFP and L10-YFP, K determined for the samples treated with filipin (K = 887) was less than one-fourth that of untreated cells (Fig. 5, A and B), and this difference was statistically significant (blue asterisk). Furthermore, the forced fit in Supplemental Fig. 1 (bottom row) substantiates the notion that these separate K values reflect distinct fits to Equation 4. Finally, Fig. 5 (B and C) also shows that membrane rafts and the actin cytoskeleton together are sufficient to account for co-clustering of L10-CFP with either L10-YFP or LAT36-YFP. Specifically, co-treatment with Lat B and filipin resulted in values for K that approach the maximum acceptor intensity, and this is indicative of a random distribution of donor and acceptor molecules (13).

Because co-clustering of the raft-associated probes was actin-dependent, we hypothesized that stabilizing the actin cytoskeleton using the compound jasplakinolide (31) would enhance the observed co-clustering. First, in Fig. 6A are data using T cells expressing Cherry-labeled actin (Cherry-actin) and showing our experimental conditions with jasplakinolide were effective in enhancing the F-actin content of the cortical cytoskeleton (Fig. 6A). Next, we observed that treating T cells with jasplakinolide caused a cholesterol-dependent increase in the co-clustering of L10-CFP and L10-YFP (Fig. 6, B and C). For example, K determined for the jasplakinolide-treated samples was less than one-fourth that of untreated cells (K = 57 versus 263). However, co-treating the samples with filipin and jasplakinolide increased K to a value ~10-fold greater than that of the samples treated with jasplakinolide alone.

Interestingly, enhancement of donor and acceptor co-clustering by jasplakinolide was not entirely specific to the L10-CFP and L10-YFP, because the drug treatment also increased the co-clustering of S15-CFP and S15-YFP or S15-CFP and S15-YFP, as well as the peripheral labeling by Cherry-actin relative to the total fluorescence signal in the cell was measured in both untreated and jasplakinolide-treated cells, and the results are represented by the bar graph (± S.E.) (right) (n = 30, and *** , p < 0.001 by Student’s t test). B, graphs showing fitted curves and accompanying residual differences from measurement of FRET between L10-CFP and either L10-YFP (left), S15-YFP (middle), or S15-CFP and S15-YFP (right). The cells were pretreated with jasplakinolide (top) or filipin after jasplakinolide treatment (bottom). The values for K ± S.E. determined in each set of conditions are plotted in C. Note the different ordinate scales. K ± S.E. = 57 ± 13 (L10-CFP/L10-YFP), 406 ± 31 (L10-CFP/S15-YFP), and 528 ± 43 (S15-CFP/S15-YFP). The values for K in the untreated samples are the same as that presented in Fig. 3. An asterisk indicates a probability of >99.9% that the K values from treated samples are different from the untreated control sample. ns represents not significantly different from the control.

**Cholesterol- and Actin-dependent Regulation of Lck in T Cells**—We and others have previously shown the raft-associated pool of Lck is down-regulated relative to that in the nonraft membrane fraction, represented by greater phosphorylation of its regulatory Tyr505 and a corresponding lower in vitro kinase activity (28, 29). The discrete regulation of raft Lck has been attributed to its sequestering from the membrane phosphatase CD45, because CD45 is excluded from rafts, and it activates Lck by dephosphorylating Tyr505. Regulation of raft-associated Lck may also be achieved by phosphorylation of Tyr505 by Csk, which associates with rafts through binding to Csk-binding protein or phosphoprotein associated with glycolipid-enriched membranes (46, 47).

Based on the results from our FRET measurements, we predicted an actin- and cholesterol-dependent regulation of Lck by
promoting sequestering of Lck from the nonraft CD45. To test this hypothesis, we measured the phosphorylation of Tyr\(^{505}\) of Lck in Jurkat cells treated with filipin, Lat B, or both, each of which affected association of Lck with the detergent-resistant raft fraction (Fig. 7A). Consistent with our hypothesis, both the filipin and Lat B decreased the Tyr\(^{505}\) content of the Lck relative to that of the untreated control (Fig. 7B). Furthermore, the greatest decrease in Tyr\(^{505}\) phosphorylation occurred in the samples co-treated with filipin and Lat B. This corresponds to conditions where the raft-associated probes exhibit a random configuration in the plasma membrane (Fig. 5) and where we anticipate the accessibility to CD45 to be the greatest.

To determine whether the changes in Tyr\(^{505}\) phosphorylation evidenced in Fig. 7B are CD45-dependent, we next measured the Lck in J45.01 cells, which are a CD45-deficient clone of Jurkat cells. In contrast to the results with the wild type Jurkat cells, neither the Lat B, filipin, nor both significantly affected phosphorylation of Lck Tyr\(^{505}\) in J45.01 cells (Fig. 7C). These data in Fig. 7 (B and C) together are therefore consistent with a cholesterol- and actin-dependent regulation of Lck by sequestering from CD45. This sequestering could occur by the actin- and cholesterol-dependent co-clustering of raft proteins, such as that identified by FRET of the membrane-anchored FPs.

**DISCUSSION**

Cell membranes are dynamic structures, represented in part by a facile mobility of protein and lipid molecules through the bilayer. Yet they retain a compartmentalization that underlies a segregation of membrane-associated functions. How this lateral heterogeneity is established and maintained represents an ongoing question. Our findings show one such mechanism is through an actin-dependent co-clustering of membrane raft-associated proteins. For example, our FRET experiments show that disrupting the actin cytoskeleton by treating cells with Lat B disrupts co-clustering of raft-associated donor and acceptor fluorescent probes, in some cases more so than occurs by sequestering cholesterol with filipin. Furthermore, promoting actin polymerization using jasplakinolide enhances co-clustering of the raft-associated probes beyond that of untreated control samples.

Altogether, our findings serve as a framework for the model illustrated in Fig. 8; the specific co-clustering of the L\(_{10}\)- and LAT\(_{36}\)-anchored probes reflects their co-association with cholesterol- and actin-dependent raft domains. Accordingly, we hypothesize that the decrease in this co-clustering by Lat B and fillipin results from disruption of the domains by these agents. We also predict that association of the L\(_{10}\)- and LAT\(_{36}\)-FPs with the domains sequesters these proteins from those in the nonraft environment. In this manner, the Lat B- and filipin-dependent increases in co-clustering of the L\(_{10}\)- and LAT\(_{36}\)-FPs with S\(_{15}\)-FP result from a loss of this sequestering by either drug treatment. Similarly, our model suggests that the apparent random distribution of the donor and acceptor pairs in cells co-treated with Lat B and filipin reflects a more effective disruption of the domains in these conditions. Treatment with jasplakinolide increased co-clustering of the L\(_{10}\)-anchored FPs in a cholesterol-dependent manner. Accordingly, we interpret this change as evidence of a jasplakinolide-dependent enhancement of the raft domains, likely through enhancement of the actin cytoskeleton by the drug treatment. In contrast, the increase in co-clustering between raft and nonraft probes L\(_{10}\)-CFP and S\(_{15}\)-YFP by jasplakinolide was cholesterol-independent and therefore likely occurred by mechanisms unrelated to the cholesterol-dependent domains.

Earlier studies demonstrated that an association of F-actin with plasma membrane complexes enriched with raft-associated proteins and lipids (32, 48). Our data reported here suggest that this association participates in establishing the raft domains. Interestingly, actin filaments bound to bilayers will order the underlying lipids (49), and this event may establish the liquid-ordered phase lipids that others hypothesize compose the membrane rafts (50). Furthermore, the composition of the detergent-resistant raft fraction suggests mechanisms by which actin filaments associate with membrane rafts (32). Notable examples include raft-associated proteins that either tether directly to actin filaments, or through intermediate proteins such as ezrin. Another example is PIP\(_2\), which is an important co-factor for many pro-
FRET Detection of an Actin-dependent Clustering of Raft Proteins

FIGURE 8. Model for cholesterol- and actin-dependent properties of raft protein co-clustering. The parameter $k$ is a relative measure of the propensity of the donor and acceptor to co-cluster. Accordingly, the elevated co-clustering evidenced with the L$_{10}$- and LAT$_{36}$-anchored FPs (dark rectangles) reflects their co-association in cholesterol and actin-dependent membrane raft domains, where they are sequestered from the S$_{15}$-FP (light ovals). Either disruption of the actin cytoskeleton with Lat B or binding of cholesterol with filipin (bottom left) perturbs the domains and associated protein co-clustering. These events also disrupt sequestering of the L$_{10}$- and LAT$_{36}$-anchored probes from S$_{15}$-FP, resulting in an increase in their co-clustering in non-raft pools of the membrane. Co-treatment with Lat B and filipin disrupts (bottom middle) residual co-clustering that remains after either treatment alone, resulting of a distribution void of co-clustering. Finally, enrichment of the cortical cytoskeleton using jasplakinolide (Jasp) promotes formation of the domains, enhancing the co-clustering of raft-associated probes. The jasplakinolide also increases a cholesterol-independent co-clustering of raft and nonraft markers, such as through corralling by the actin filaments that occur because of the jasplakinolide. Finally, experiments with Lck show that conditions that affect the relative co-clustering of raft and nonraft probes function toward regulation of raft-associated enzymes.

proteins that function in actin polymerization and which is enriched in the detergent-resistant raft fraction (51). Consistent with the notion that raft-associated PIP$_2$ is important for interactions between membrane rafts and actin filaments, earlier studies showed that co-localization of PIP$_2$ with membrane raft markers also functioned in cytoskeleton-dependent membrane patching and protrusion of the plasma membrane (52), and actin-driven vesicle translocation in the cytoplasm (53). However, these findings occurred in cells other than Jurkat T cells, and whether these events represent localization of PIP$_2$-dependent functions to membrane rafts also remains to be resolved. In summary, many factors likely underlie association between the actin cytoskeleton and membrane raft complexes, and further study is necessary to better define the nature of these interactions and their role in establishing these domains.

Another interpretation of our data regarding the actin-dependent co-clustering of raft-associated FPs is that this event arises from corollaring of raft proteins by underlying actin “fences.” For example, the cortical cytoskeleton is a highly reticulated network of actin filaments that forms a meshwork of fibers adjoining the inner leaflet of the plasma membrane (54). Furthermore, molecular mobility measurements show that the actin filaments transiently confine membrane proteins and lipids within actin corals established by the actin cytoskeleton (55, 56). Such a corollaring could promote protein clustering and may account for the cholesterol-independent increase in co-clustering of L$_{10}$-CFP and S$_{15}$-YFP measured in the jasplakinolide-treated cells. However, it is difficult to envision how such a mechanism would account for the co-clustering that was specific to the raft-associated molecules, as well as the cholesterol-dependent nature of this co-clustering. Accordingly, we favor the model where the co-clustering occurs through an actin-dependent change in the membrane environment that favors in particular the raft-associated proteins.

An additional finding was that disrupting protein co-clustering using Lat B had no effect on the association of L$_{10}$-GFP with the detergent-resistant raft fraction (Fig. 4) and only a marginal effect on Lck (Fig. 7A). These results underscore the caution that one should exercise in interpreting membrane fractionation data. Because membrane rafts are heterogeneous in nature, it is difficult to predict the size and composition of the domains represented by the detergent-insoluble raft fraction. In our experiment with Lat B, the decrease in protein co-clustering while maintaining detergent insolubility may represent a dispersion of raft microdomains into nanoclusters because the latter are too small to accommodate significant protein co-clustering as they contain at most two or three protein molecules (14).

Related to the issue of detergent insolubility, association of proteins with detergent-resistant membranes is also likely to be cell type-specific. For example, one recent study showed that a sizable fraction of the S$_{15}$-labeled probes occurs in the detergent-resistant fraction in B cells (17). This contrasts with our results with Jurkat cells (Fig. 1) and other types of T cells (data not shown), because we detect no more than 1–2% of the protein in the raft fraction. The factors governing association of proteins and lipids with detergent-resistant membranes are complex, and it is therefore reasonable to conclude that different cell types will have separate properties in this regard. Importantly, our results reported here using FRET are consistent with exclusion of the S$_{15}$-FPs from membrane rafts containing either the L$_{10}$- or LAT$_{36}$-anchored molecules.

The actin cytoskeleton is an adaptable structure, undergoing reorganization in response to extracellular cues. Furthermore, the dynamic nature of the actin cytoskeleton may serve to promote and regulate protein co-associations in the plasma membrane to enhance or inhibit signaling pathways. Consistent with this hypothesis, we observed an actin-dependent regulation of the Src family kinase Lck, indicated by an inhibition of phosphorylation of Tyr$^{505}$ by treating cells with Lat B. Furthermore, the effect of Lat B on the Tyr$^{505}$ phosphorylation required CD45 because it was not observed in J45.01 cells. In an earlier study, it was surmised that down-regulation of raft-associated Lck is due to its sequestering from CD45 (28), the latter being excluded from the raft fraction. Our findings here extend this earlier work by showing that the actin cytoskeleton is likely instrumental toward establishing this sequestering.

Actin polymerization and remodeling of the cytoskeleton are integral to many of the events that occur on the surface of the...
cell, such as cell motility, adhesion, and cell signaling. These processes often utilize proteins that associate with membrane rafts, and this accounts for the co-capping of rafts and actin filaments in the plasma membrane such as occurs in activation of lymphocytes (16, 22). Our data show that a synergy exists between the actin filaments and the membrane cholesterol, where both elements are necessary to achieve efficient co-clustering between proteins that occur in the rafts. Future studies will better define the mechanism by which this synergy between membrane rafts and the actin cytoskeleton occurs.

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