T Cell Signal Regulation by the Actin Cytoskeleton*\[S\]

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T cells form an immunological synapse (IS) that sustains and regulates signals for cell stimulation. Enriched in the IS is the Src family kinase Lck. Conversely, the membrane phosphatase CD45, which activates Src family kinases, is excluded, and this is necessary to avoid quenching of T cell receptor phosphosignals. Data suggest that this arrangement occurs by an enrichment of cholesterol-dependent rafts in the IS. However, the role of rafts in structuring the IS remains unclear. To address this question, we used fluorescence resonance energy transfer (FRET) to interrogate the nanoscopic structure of the IS. The FRET probes consisted of membrane-anchored fluorescent proteins with distinct affinities for rafts. Both the raft and nonraft probes exhibited clustering in the IS. However, co-clustering of raft donor-acceptor pairs was 10-fold greater than co-clustering of raft-nonraft pairs. We measured the effect of disrupting rafts in the IS on CD45 localization and Lck regulation by treating stimulated T cells with filipin. The filipin specifically disrupted co-clustering of the raft FRET pairs in the IS and allowed targeting of CD45 and this is necessary to avoid quenching of T cell receptor phosphosignals at the TCR complex (15, 16).

Despite much study, the mechanism by which CD45 is segregated from the IS is poorly understood and even controversial. One model relates to the cholesterol-dependent membrane rafts. Rafts are postulated to be discrete membrane domains that are composed of proteins and lipids that occur in a detergent-resistant membrane (DRM) fraction present in cell lysates (17). Data that show a discrete and cholesterol-dependent clustering of DRM-associated molecules (18–24) are consistent with this model. Many of the proteins and lipids enriched in the IS also occur in DRMs (25–28), suggesting that the IS represents a specialized raft compartment in the plasma membrane (29). Similarly, CD45 is largely excluded from DRMs (30–32), thus coinciding with its exclusion from the IS.

Although the IS has a protein and lipid composition that is consistent with it being a raft-enriched compartment, other data contrast with this model. Examples include protein localization in the IS despite a low affinity for DRMs (33, 34) and similar rates of diffusion in the IS for both DRM-resident and DRM-excluded proteins (35). Protein size is also an important determinant for localization in the IS (36–38), and the “kinetic segregation model” hypothesizes that the composition of the IS is established by whether the ectodomain of proteins fit into the space between the T cell and APC (39). CD45 is predicted to belong to the category of proteins that are too large for the intercellular space. Although the membrane raft and kinetic segregation models are not mutually exclusive, the relative contribution of each in protein targeting to the IS for T cell activation is not clear.
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Much of the uncertainty regarding the mechanisms that define IS macrostructure, such as CD45 exclusion, relate to a poor understanding of its plasma membrane nanostructure. This is especially a shortcoming in models relating to rafts because most occur as nanoscale domains (20, 24, 40). Measurement of FRET between membrane-associated proteins labeled with donor and acceptor fluorophores is one method to detect nanoscale interactions influenced by membrane heterogeneities, such as rafts. FRET measurements of labeled DRM markers show nanoscale clustering of DRM-resident proteins and lipids (19, 23, 41, 42). We recently showed using FRET a specific co-clustering of DRM-associated probes in the plasma membrane that was sensitive to compounds that either sequester cholesterol or alter the cortical actin cytoskeleton (43). Conditions that disrupted DRM-associated probe clustering resulted in deregulation of Lck by dephosphorylation of its Tyr^505. These data suggest that rafts are structured by actin filaments that attach to the plasma membrane, producing domains that function in Lck regulation (43). The IS is enriched with F-actin, but whether the cytoskeleton structures rafts in the IS for Lck regulation has not been shown.

We report experiments that show nanoscopic clustering of membrane reporters in the IS by measuring FRET between membrane-anchored fluorescent proteins (FPs). We include in our measurements ISs treated to disrupt cholesterol- and cytoskeleton-dependent properties. We relate the effect of these treatments on IS nanostructure to the biological properties of the IS by measuring the localization of CD45 and its substrate, Lck Tyr(P)^505. We report findings that show exclusion of CD45 from the IS that occurs by structuring of rafts at the cell interface. We show that these properties are necessary for maintaining regulation of Lck in the IS.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Jurkat T cells (clone E6-1) and Raji B cells were maintained in medium containing RPMI 1640 supplemented with antibiotics and 10% fetal bovine serum at 37 °C in the presence of 5% CO2. Jurkat cells were maintained in RPMI-HEPES containing 5 μM jasplakinolide (Invitrogen), or 5 μg/ml filipin (Cayman Chemicals, Ann Arbor, MI). Samples treated with media containing vehicle alone served as a control. All incubations were for 30 min at 37 °C.

Fluorescence Labeling of Cells—Fixed cell conjugates were immunostained using primary antibodies and Texas Red-conjugated secondary antibodies. For experiments including staining of intracellular proteins, the fixed samples were permeabilized using 0.2% Triton X-100 in phosphate-buffered saline. Lck Tyr^505 (Cell Signaling Technology, Danvers, MA) and PKC-θ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were stained with rabbit polyclonal antibodies. Lck, CD3e (UCH-T1), and CD45 (HI30) (Santa Cruz Biotechnology, Inc.) were stained using mouse monoclonal antibodies.

T cells were labeled with Laurdan (6-dodecanoyl-2-dimethylamino-naphthalene, Invitrogen) by incubating 2 × 10^5 cells for 45 min at 37 °C in 0.5 ml of RPMI-HEPES containing 5 μM Laurdan. Raji B cells were labeled with CellTracker™ Orange CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine)) in RPMI-HEPES for 15 min at 37 °C containing 4 μM dye. Labeling with Laurdan and CellTracker™ Orange CMTMR were performed before cell conjugation.

Cell Imaging and Analysis—Fluorescent protein imaging was performed using a Zeiss LSM 510 META confocal microscope (Oklahoma Medical Research Foundation imaging core facility). CFP was excited at 458 nm, and emission was detected between 473 and 505 nm. YFP was excited at 514 nm and detected at 530–600 nm. Images were collected using a ×63 objective (numerical aperture 1.2), and recorded in 12-bit mode at a scan rate of 2.56 μs/pixel. Laurdan and CellTracker™ Orange CMTMR imaging was performed using a Leica SP2 multiphoton microscope equipped with a ×63 Plan APO 1.4 numerical aperture objective. Laurdan fluorescence was excited using an 800-nm laser line. Laurdan fluorescence was imaged using 400–460-nm and 470–530-nm bandwidths. To image CellTracker™ Orange CMTMR, the samples were excited at 543 nm and detected at 550–620 nm. All image processing and quantitation was performed using iVision 4.0 image analysis software (Bio Vision).

FRET measurements were performed by acceptor photobleaching as we have described (22). Briefly, YFP was photobleached in a 8 × 5-pixel (2.1 × 1.3-μm) region of interest (ROI) in the plasma membrane using a 514-nm laser line at full power. Prebleach (pre) and post-bleach (post) images were recorded for both CFP and YFP channels. Cells expressing CFP alone were taken through all of the steps of FRET measurement to correct for bleaching of CFP during YFP bleaching (CFP<sub>YFP bleached</sub>). A correction factor for YFP bleed-through to the CFP channel (CFP<sub>YFP bleedthrough</sub>) was generated using cells sedimented by centrifuging (Eppendorf) for 2 min at 1500 rpm, and then incubated at 37 °C for 30 min. Following conjugation, the samples were seeded onto poly-1-lysine (Sigma)-coated coverslips and fixed using 2% paraformaldehyde. For the drug treatments, conjugates were first seeded onto coverslips, washed with RPMI containing 50 mM HEPES (pH 7.4) (RPMI-HEPES), and then incubated in RPMI-HEPES containing either 5 μM latrunculin B (Lat B) (Calbiochem), 0.5 μM jasplakinolide (Invitrogen), or 5 μg/ml filipin (Cayman Chemicals, Ann Arbor, MI). Samples treated with media containing vehicle alone served as a control. All incubations were for 30 min at 37 °C.
expressing YFP alone and imaged in the CFP channel. Fluorescence intensities of each ROI were measured on a pixel-by-pixel basis, and measurement of YFP$_{pre}$ - YFP$_{post}$ showed that the photobleaching extinguished $>95\%$ of the YFP fluorescence. Consistent with recent findings, photobleaching of cells expressing YFP alone failed to show any photoconversion of YFP to a CFP-like species (44).

CFP$_{pre}$ was corrected for YFP bleed-through into the CFP channel using the following operation,

$$\text{CFP}_{\text{pre, corrected}} = \text{CFP}_{\text{pre}} - \text{CFP,YFP bleach}$$

(Eq. 1)

CFP$_{\text{post,bleach}}$ was corrected for both bleaching of CFP during YFP bleaching and YFP bleed-through by the following,

$$\text{CFP}_{\text{post, corrected}} = (\text{CFP}_{\text{post}} - \text{CFP,YFP bleach}) + \text{CFP,YFP bleach}$$

(Eq. 2)

FRET efficiency ($E\%$) was calculated using Equation 3.

$$E\% = \frac{(\text{CFP}_{\text{post, corrected}} - \text{CFP}_{\text{pre, corrected}})}{\text{CFP}_{\text{post, corrected}}} \times 100$$

(Eq. 3)

FRET was measured in 75–100 separate conjugates for each sample. Measurements were restricted to cells where the donor/acceptor ratio was $\sim 1.0$, calculated using the ratio YFP$_{pre}$/CFP$_{\text{post, corrected}}$. Co-clustering was measured by fitting plots of $E\%$ versus acceptor intensity to the following isothermal binding equation (23),

$$E\% = \frac{E_{\text{max}} F}{(F + K)}$$

(Eq. 4)

where $F$ represents the fluorescence intensity of the acceptor, which we defined as the prebleach intensity of the YFP fluorescence. Curve fitting and all statistical analysis were performed using Igor Pro (WaveMetrics, Lake Oswego, OR), and error analysis shows this to produce unique values for $K$ from combined sets of FRET data (22). The statistical significance of differences in $K$ values was determined using a two-tailed Student’s $t$ test for distributions of unequal variance as described previously (22). $K$ values determined to be significantly different are indicated by an asterisk, which represents a probability greater than 99.99% that the two values are different.

Measurement of relative fluorescence enrichment of proteins in the IS was performed by first identifying T cell-B cell conjugates using a differential interference contrast (DIC) image of each field. The IS was defined as the interface between the Jurkat T cell and SEE-pulsed Raji B cell. Thus, following background subtraction, T cell plasma membrane in the contact site and plasma membrane outside of the contact site were outlined separately, and the average fluorescence intensity of each region was measured. The relative fluorescence enrichment at the contact site was calculated by taking the ratio of average fluorescence intensity at the contact site over the average fluorescence intensity of the remaining plasma membrane.

Generalized polarization (GP) of Laurdan was calculated using the following equation (45),

$$\text{GP} = \frac{l_{400-460} - G \times l_{470-530}}{l_{400-460} + G \times l_{470-530}}$$

(Eq. 5)

where $G$ represents a correction factor for the experimental setup. The $G$-factor was calculated as described (46) using a standard solution of 0.5 $\mu$m Laurdan in DMSO.

Electron Microscopy—Jurkat-SEE-pulsed Raji cell conjugates were seeded onto poly-l-lysine-coated glass coverslips. Cells were fixed with 2% glutaraldehyde in 100 mm cacodylate buffer for 1 h at room temperature. After washing with cacodylate buffer, cells were stained with saturated uranyl acetate for 1 h followed by osmium tetroxide for 1 h at room temperature. The cells were embedded in resin, and ultrathin sections were cut using an ultramicrotome (Reichert UltracutE). The sections were imaged by transmission electron microscopy (Hitachi H-7600). Samples were initially screened at $\times 3000$ magnification, and digital images ($2000 \times 2000$ pixels) were captured using an in-line digital camera (Eastman Kodak Co.) at $\times 35000$ magnification. Intermembrane distance measurements of the acquired images were performed using iVision 4.0 image analysis software. Intermembrane distances were measured in regions where membranes were aligned parallel to each other. For each synapse, measurements were taken from more than 15 locations at the cell interface.

RESULTS

Localization of Membrane-anchored Fluorescent Proteins to the T Cell IS—We illustrate in Fig. 1A the membrane-anchored FPs that we used for labeling the raft and nonraft fractions of the plasma membrane. In brief, CFP, GFP, and YFP were targeted to the plasma membrane by either the S15 sequence restricts and a cholesterol-dependent co-clustering in the plasma membrane (22, 47, 48). In contrast, the S15 sequence restricts FPs to the nonraft, detergent-soluble membrane fraction; S15-anchored FPs exhibit a cholesterol-independent co-clustering that is weaker than that between the raft-associated probes (22).

To study the properties of the membrane-anchored FPs in the IS, we stimulated labeled Jurkat T cells with SEE pulsed Raji B cells. Fig. 1, B and C, show confocal images of labeled conjugates and, in Fig. 1D, are the relative fluorescence enrichment (Rel. Fluor. Enrich.) values that we measured for each label in the IS of many conjugates. The enrichment values were calculated by dividing the average fluorescence intensity of the plasma membrane at the cell interface by the average intensity of the remaining plasma membrane. Because CD45 is excluded from the IS (16), we controlled for the specificity of the IS labeling by immunostaining conjugates with antibody to CD45. These data show that the stimulation produced an IS marked by an enrichment of Cherry-labeled actin and the raft probes L10-GFP and LAT36-YFP. Nonraft S15-YFP was present in the IS but not enriched. Accordingly, the average relative fluorescence enrichment value for S15-YFP was $\sim 1.0$, significantly greater than that.
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FIGURE 1. Targeting of membrane-anchored fluorescent proteins to the IS. A, minimal signals for targeting FPs to the plasma membrane. 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 includes the transmembrane domain of LAT (box) and a pair of membrane-proximal cysteine residues. Sites that are either myristoylated (Myr) or palmitoylated (Palm) are indicated. L$_{10}$ and LAT$_{36}$ signals target proteins to the raft fraction, and the S$_{15}$ sequence restricts proteins to the nonraft fraction. B, confocal (left, middle) and DIC (right) images of a Jurkat T cell co-expressing L$_{10}$-GFP and Cherry-labeled actin (Cherry-Actin) and conjugated to an SEE-pulsed Raji B cell. Fluorescence intensity is represented by pseudocolor, and the color assignment for intensity level is shown in the accompanying scale. The asterisks indicate the position of the B cell. C, confocal and DIC images of Jurkat T cells conjugated to SEE-pulsed B cells. The T cells were labeled with L$_{10}$-, LAT$_{36}$-, or S$_{15}$-FP, or immunostained with antibody to CD45 and Texas Red (TX Red)-conjugated secondary antibody. D, the relative fluorescence enrichment (Rel. Fl. Enrich.) in the IS for the indicated proteins, calculated by dividing the average fluorescence intensity at the IS by the average fluorescence intensity of the remaining plasma membrane. IS was defined as the contact area between a Jurkat T cell and the SEE-pulsed Raji B cell. Each point represents measurements of a single conjugate. The mean and S.E. of each sample are indicated by the red and black bars, respectively. ***, p < 0.0001; **, p < 0.001; ns, not significant, as determined by Student’s t test. Scale bars, 5 μm.

The distinct properties of S$_{15}$-FP and CD45 in the IS suggest that factors other than restriction from rafts contribute to protein exclusion from the IS.

Raft-associated Fluorescent Proteins Exhibit Elevated Co-clustering in the IS—We next measured the co-clustering of the membrane-anchored FPs in the IS by imaging FRET between the CFP- and YFP-labeled forms of L$_{10}$-, LAT$_{36}$-, and S$_{15}$-FP. We measured FRET as described (see “Experimental Procedures”) by determining the increase in donor (CFP) fluorescence following photobleaching of the acceptor (YFP) in fixed cells. We previously showed, using control CFP-YFP fusion proteins, that the increase in CFP fluorescence by YFP photobleaching is specific to FRET (22). Therefore, we asked if co-clustering of the membrane raft markers varied between the different regions of the IS. Accordingly, we measured the FRET between L$_{10}$-CFP and L$_{10}$-YFP in the c-SMAC, which we identified by immunostaining conjugates with antibody to CD3ε (supplemental Fig. 2). For the p-SMAC, we measured a region of the cell interface that was adjacent to the CD3ε staining. In Fig. 2C, the fitted curves for each set of measurements, and we summarize the K values from these measurements in Fig. 2B. These data show that values for K from FRET between L$_{10}$-CFP and L$_{10}$-YFP were similar for the c-SMAC and p-SMAC, indicating that their associative properties did not vary significantly between the different regions of the IS.

To determine if co-clustering of the probes in the IS is distinct from that of remaining plasma membrane, we measured FRET between L$_{10}$-CFP and either L$_{15}$, LAT$_{36}$, or S$_{15}$-YFP in non-IS plasma membrane. The determined values for K and corresponding fitted curves are shown in Fig. 2D and (22, 23). Measuring FRET based on sensitized emission of YFP following CFP excitation in live cells at 37 °C demonstrated a similar discrete and specific co-clustering of the raft probes absent fixation (supplemental Fig. 1).

Shown in Fig. 2A are curve fittings to Equation 4, using FRET measured in the IS of Jurkat cells conjugated to SEE-pulsed B cells. The donor in each experiment was L$_{10}$-CFP, and the acceptor was either L$_{10}$-YFP (left) or S$_{15}$-YFP (right). Note that each point represents measurement of a single ROI in a separate conjugate. The red lines in Fig. 2A and elsewhere represent the fitted curves, and the blue dashed lines are the boundaries for the 95% confidence level for each curve fitting. The residuals (top) are random and close to zero, indicating a favorable fit of the experimental data to Equation 4. The values for K from the two sets of measurements are plotted in Fig. 2B, where the asterisk represents a 99.99% certainty that the indicated values are statistically different as determined by Student’s t test for distributions of unequal variance. These data show a 10-fold greater co-clustering of the L$_{10}$-anchored FPs in the IS over that of L$_{10}$-CFP and S$_{15}$-YFP.

Thus, co-clustering in the IS was greatest when both the donor and acceptor associated with rafts.

Co-localization measurements showed that the L$_{10}$-anchored FPs and labeled actin occur in both the c-SMAC and p-SMAC of the IS (supplemental Fig. 2). We therefore next asked if co-clustering of the membrane raft markers varied between the different regions of the IS. Accordingly, we measured the FRET between L$_{10}$-CFP and L$_{10}$-YFP in the c-SMAC, which we identified by immunostaining conjugates with antibody to CD3ε (supplemental Fig. 2). For the p-SMAC, we measured a region of the cell interface that was adjacent to the CD3ε staining. In Fig. 2C, the fitted curves for each set of measurements, and we summarize the K values from these measurements in Fig. 2B. These data show that values for K from FRET between L$_{10}$-CFP and L$_{10}$-YFP were similar for the c-SMAC and p-SMAC, indicating that their associative properties did not vary significantly between the different regions of the IS.

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supplemental Fig. 3, respectively. These data show that co-clustering of the raft-associated donor-acceptor pairs was ~8-fold greater in the IS than that of non-IS plasma membrane. In contrast, co-clustering of L10-CFP and S15-YFP did not differ significantly between the separate regions. Also listed in the legend to Fig. 2 are K values determined for the plasma membrane of unstimulated cells. These data show that co-clustering of the raft probes in unstimulated cells was distinct from that of both IS and non-IS plasma membrane of stimulated cells. Thus, stimulation caused global changes in raft probe co-clustering to produce a distinct and specific enhancement of their co-clustering in the IS.

Specific Co-clustering of L10-anchored FPs in the IS by Cholesterol and the Actin Cytoskeleton—To determine if probe co-clustering in the IS is cholesterol-dependent, we measured the IS of conjugates that were treated with filipin to sequester membrane cholesterol. The filipin was added to the samples following establishment of an IS by stimulating the T cells with SEE-pulsed B cells. This treatment typically disrupts T cell-APC conjugates, frustrating attempts to measure parameters affecting plasma membrane structure in the IS. However, seeding conjugates onto poly-L-lysine-coated glass before treatment maintained binding of the T cells to the APCs without affecting the intercellular distance. We show this in Fig. 3 by transmission electron microscopy of untreated and filipin-treated conjugates. The T cells in the conjugates were identified by immunogold labeling with an antibody to CD3ε. Representative examples of the T cell-B cell interface are in Fig. 3B, and quantitation of the distance between the outer membranes at the IS is shown in Fig. 3C. Each point in Fig. 3C represents the average of 15–20 separate measurements in a single conjugate. These data show that no detectable change in the distance between the cells occurred by the filipin. The measured distances in control ISs are in agreement with the previously reported data (37).

Confocal imaging and FRET measurements showed that treatment of conjugates with filipin disrupted both the enrichment (Fig. 4, A and B) and co-clustering of the L10-anchored probes in the IS (Fig. 4C and supplemental Fig. 4). A similar effect occurred using the oxysterol 7-ketocholesterol, which disrupts rafts without sequestering or removing cholesterol (49) (supplemental Fig. 5). Conversely, filipin did not significantly affect K determined from FRET between L10-CFP and S15-YFP (Fig. 4C), showing that the cholesterol-dependent co-clustering was specific for raft-associated donor-acceptor pairs.

Chemical inhibitors that disrupt the cytoskeleton also disrupt clustering of raft markers (reviewed in Ref. 43). As one example, we recently showed using FRET that plasma membrane co-clustering of the L10-FPs is disrupted by Lat B but not co-clustering of L10- and S15-FP (22). Inhibitor concentrations that disrupt raft protein clustering also inhibit proximal and distal signaling from the TCR (50, 51), suggesting a role for cytoskeleton-dependent rafts in T cell signaling. Accordingly, we tested the role of the actin cytoskeleton in DRM protein clustering in the IS by measuring the IS following treatment of conjugates with Lat B. Confocal imaging showed that the Lat B disrupted capping of L10-YFP in the conjugates (Fig. 4, A and B). Furthermore, K determined from FRET between L10-CFP and L10-YFP was similar to that of the maximum acceptor intensity in the plot of E% versus acceptor intensity (Fig. 4D, top). This is indicative of a ran-
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FIGURE 3. Visualization and measurement of the T cell-APC interface in untreated and treated conjugates. Conjugates containing Jurkat T cells stimulated with pulsed Raji cells were seeded on to poly-L-lysine-coated glass and then incubated with either buffer plus vehicle alone or containing filipin. A third set was pretreated with jasplakinolide before the addition of filipin. The samples were next prepared and imaged by electron microscopy to measure the T cell-APC interface (A). Shown is a representative example of a T cell conjugated to a SE-pulsed B cell, ×2000. B, regions of the interface between conjugates that were either untreated, treated with filipin alone, or treated with jasplakinolide followed by filipin (×35,000). C, average intermembrane distances at the IS of Jurkat T cell and SEE-pulsed B cell. The distance between the cells was measured at the cell interface using ×35,000 magnification images. Each point represents the average of >15 measurements of the intermembrane distance in an individual conjugate. ns, not significant.

Because both membrane cholesterol and the actin cytoskeleton were important for enrichment and co-clustering of the L10-anchored probes in the IS, we asked if blocking the actin depolymerization using jasplakinolide affected the filipin-dependent changes in these properties. For this experiment, conjugates were first treated with jasplakinolide, followed by a second treatment using filipin. Imaging by electron microscopy showed that the intercellular distance at the T cell-APC interface was maintained following this set of treatments (Fig. 3, B and C). Interestingly, L10-FP enrichment and co-clustering in the IS were maintained in these conditions despite the treatment with filipin (Fig. 4, A–C, and supplemental Fig. 4). Fluorescence imaging of filipin fluorescence showed efficient labeling of the IS (supplemental Fig. 6). Thus, the blocking of filipin-dependent changes in the IS by jasplakinolide was not due to an inhibition of the filipin targeting to the IS. These data show that jasplakinolide minimizes the role of cholesterol in L10-FP enrichment and co-clustering in the IS.

**Jasplakinolide Blocks Lipid Decondensation in the IS by Filipin**—One interpretation of the results in Fig. 4 is that the jasplakinolide maintains a distinct lipid environment of the IS in the presence of filipin that favors co-clustering of the raft-associated probes. To test this hypothesis, we used the lipophilic reporter Laurdan to measure the effect of treatment with jasplakinolide and filipin on lipid condensation in the IS, which is indicative of lipid ordering. Laurdan undergoes a blue shift in its emission spectrum from being centered near 500 nm in loosely packed fluid phase lipids to centered near 440 nm in tightly packed liquid-ordered phase lipids (52). Lipid condensation in the bilayer is quantitated using the GP of Laurdan fluorescence (45), which is the normalized ratio of its fluorescence centered at 440 and 500 nm (Equation 5). GP values range between 1.0 and 1.0, with higher values indicating greater lipid ordering. GP values are elevated in the IS relative to non-IS plasma membrane (53).

**FIGURE 4. Cholesterol- and cytoskeleton-dependent properties of probe co-clustering in the IS.** A, confocal (left) and DIC (right) images of conjugated Jurkat T cells expressing L10-YFP. The conjugates were untreated or treated with either filipin, Lat B, or jasplakinolide followed by filipin. All of the treatments were for 30 min at 37 °C, followed by fixation. The asterisks in the confocal images indicate the position of the B cell. White bars, 5 μm. B, relative fluorescence enrichment values for L10-YFP in the IS in the indicated conditions. **, p < 0.001 by Student’s t test. The data for the untreated samples are the same that are shown for the L10-YFP sample in Fig. 1D. C, values for K from FRET between either L10-CFP and L10-YFP or S15-CFP and S15-YFP following the indicated treatments. All measurements were in the IS. The values for the L10-CFP and L10-YFP pair are 80, 602, and 93 for the untreated, filipin-alone, and jasplakinolide followed by filipin-treated, respectively. For the L10-CFP and S15-YFP pair, the values are 877, 756, and 671 for untreated, filipin-alone, and jasplakinolide followed by filipin, respectively. D, fitted curves to Equation 4 from FRET between L10-CFP and L10-YFP measured in the IS of T cells conjugated to SEE-pulsed B cells and treated with Lat B (top). For comparison, we show the fitted curve from Fig. 2A representing curve fitting from FRET between L10-CFP and L10-YFP in the IS of untreated conjugates (bottom).
B cell plasma membrane occurred after the B cells conjugated to Laurdan-labeled T cells. We therefore measured the Laurdan fluorescence in the IS with the understanding that it included both T cell and B cell plasma membrane, an acceptable approximation because the B cell plasma membrane in the IS has many of the same structural characteristics as the T cell plasma membrane (54). Because many of the T cells underwent homotypic adhesion, we identified bona fide T cell-APC conjugates by prelabeling the B cells with CellTracker™ Orange CMTMR. The GP values from ~100 separate conjugates in each set of conditions are plotted in Fig. 5B. These data show that the filipin significantly decreased the average GP value at the contact site but not when the conjugates were pretreated with jasplakinolide. Thus, jasplakinolide blocks changes in lipid condensation at the IS by filipin.

**Jasplakinolide Blocks Filipin-dependent Targeting of CD45 to the IS and Lck Deregulation**—We next asked if treatment with either filipin alone or filipin following pretreatment with jasplakinolide affected CD45 targeting and Lck regulation in the IS. We therefore immunostained conjugates with antibody to either CD45 or Lck Tyr(P)505 in each set of conditions, and we show representative confocal images in Figs. 6A and 7A. Total Lck in the IS was also measured by immunostaining conjugates with a monoclonal antibody that is specific to its N terminus (Fig. 7B). Accompanying the confocal data is the quantitation of IS labeling by each label that we measured in many labeled conjugates (Figs. 6B and 7C). As noted earlier, CD45 is typically excluded from the cell interface, and Fig. 7 shows that this was accompanied by a significant enrichment of Lck Tyr(P)505. Consistent with earlier findings (8), most of the Lck was concentrated in the center of the IS representing the c-SMAC, and this was accompanied by most of the Lck Tyr(P)505 as well. Treatment with filipin resulted in targeting of CD45 to the IS and a corresponding decrease in the Lck Tyr(P)505 signal. However, both the CD45 targeting and loss of Lck Tyr(P)505 signal by filipin were blocked when the samples were pretreated with jasplakinolide. Although filipin reduced the amount of Lck expression in the IS (Fig. 7C), this was not enough to offset the changes in Tyr(P)505 levels. Altogether, these data show that targeting of CD45 to the IS and dephosphorylation of Lck Tyr(P)505 occurs in conditions where the raft character of the IS is disrupted as measured...
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As we suggest in our model, structuring of bilayer lipids by actin filaments could occur through factors that attach the filaments to the inner leaflet of the membrane. In the IS, these factors could include ezrin, filamin, and talin, which are enriched in the IS and bind simultaneously to actin filaments and the plasma membrane (8, 58, 59). Furthermore, many actin-binding membrane proteins undergo regulation in their attachment of cytoskeleton to the plasma membrane, thus providing a mechanism for activating or inhibiting actin attachment to the IS for structuring rafts and regulating Lck.

In samples treated with jasplakinolide, we observed that the actin cytoskeleton in certain conditions could offset the effect of filipin on clustering of the raft-associated probes and their enrichment in the IS. One interpretation of this finding is that jasplakinolide lowers the threshold of cholesterol necessary to structure rafts by the cytoskeleton. Jasplakinolide may also block the decrease in raft probe clustering that occurred with 7-ketocholesterol. Interestingly, experiments with model membranes showed that actin filaments can order lipids in fluid phase bilayers (60). Jasplakinolide-treated cells may be one set of conditions where this can occur in biological systems. Consistent with this interpretation, our experiment imaging Laurdan fluorescence indicates that pretreating conjugates with jasplakinolide maintains lipid condensation at the cell interface after filipin is added. However, caution must be exercised in interpreting these Laurdan data because the probe also labels membranes other than the T cell plasma membrane, including the B cell plasma membrane. Altogether, further study is necessary to better define the hierarchy of actin- and cholesterol-dependent forces that affect protein clustering and localization in the IS.

Jasplakinolide also caused a modest yet significant increase in co-clustering of L10-CFP with S15-YFP in the IS (Fig. 4D). This property may reflect an increase in nonspecific clustering through corralling from actin filaments underlying the plasma membrane (61).

Our data also illustrate the complexity underlying protein targeting to the IS. For example, both S15-FP and CD45 are excluded from rafts, yet S15-FP occurred in the IS while CD45 was depleted. Several studies have shown that a large extracellular domain can lead to exclusion of membrane proteins from the IS, a property that has been attributed to the depletion of CD45 from the IS (37). Nevertheless, we observed targeting of CD45 to the IS in conditions where no measurable change in the distance between the cells occurred. Thus, although the limited distance between the T cell and APC may contribute to exclusion of some proteins from the IS, we showed that much of this can be overcome by changing the physical properties of the bilayer in the IS.

The probes used in this study consist of short peptide sequences that anchor FPs to the plasma membrane, a feature that minimizes the role of protein-protein interactions in probe targeting to the IS and co-clustering. Nevertheless, molecular mobility measurements show that peptide-binding sequences, such as Tyr(P) residues, can have a significant affect on protein mobility in the membrane (35). A similar effect is likely to occur in protein targeting to the IS and co-clustering in the membrane. In

FIGURE 7. Filipin-dependent deregulation of Lck in the IS is blocked by jasplakinolide. A and B, confocal (left) and DIC (right) images of T cells conjugated to SEE-pulsed B cells and treated as indicated. The conjugates were stained with antibodies specific to either the C-terminal regulatory phosphotyrosine of Lck (Tyr505) (A) or Lck (B), followed by Texas Red-labeled secondary antibody. The asterisks indicate the position of the B cell. The arrows indicate the cell contact site. The scale bars represent 5 μm. C, relative fluorescence enrichment of Tyr(P)505 or total Lck in the IS. **, p < 0.001; ***, p < 0.0001.

by co-clustering of the L10-anchored probes and Laurdan fluorescence.

DISCUSSION

We addressed in this study questions regarding membrane structure in the IS using FRET imaging to measure nanoscopic associations between membrane-anchored FPs. Our data show an enriched co-clustering in the IS that was specific for raft-associated donor-acceptor pairs and was both cholesterol- and cytoskeleton-dependent. Furthermore, conditions that disrupted raft protein clustering resulted in targeting of CD45 to the IS and dephosphorylation of Lck Tyr505. We conclude from these data that the cytoskeleton structures rafts in the IS, maintaining a membrane environment that disfavors CD45 in the IS. Exclusion of CD45 from the IS allows accumulation of Lck that is down-regulated through phosphorylation of its Tyr505. We surmise that initial phosphorylation of Tyr505 in the IS occurs by Csk (55), which is targeted to rafts through binding to Cbp (56, 57).
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FIGURE 8. A model for regulation of Lck in the IS by actin cytoskeleton. The actin cytoskeleton structures rafts (colored headgroups) in the IS, creating a membrane environment that is favorable for order-preferring proteins, such as the lipid-anchored Lck, but unfavorable for CD45. Lck is regulated by phosphorylation of its regulatory Tyr(P)505, and exclusion of CD45 from the IS results in accumulation of Lck Tyr(P)505. Treatment with filipin increases the accessibility of CD45 to Lck in the IS by disrupting rafts and allowing CD45 to target to the interface, resulting in a decrease of Lck Tyr(P)505 levels in the IS. Stabilizing the cortical actin cytoskeleton in the IS using jasplakinolide maintains the rafts in the presence of filipin, thereby also maintaining CD45 exclusion and sequestering of Lck Tyr(P)505 from CD45.

the case of Src family kinases such as Src and Lck, further study is necessary to identify the contribution of protein-binding sequences, such as Tyr(P) residues and Src homology domains, to targeting of full-length protein to the IS and co-clustering.

Recent super-resolution imaging data show the IS contains nanoscopic protein islands labeled with TCR and proximal signaling proteins (62). Interestingly, the size of the islands is sensitive to both cholesterol and the actin cytoskeleton. These super-resolution data and our results contrast with findings by Glebov and Nichols (33), which failed to detect clustering of labeled GPI-anchored proteins in stimulated T cells. Other studies have shown nanoscopic clustering of GPI-anchored proteins (18, 19, 63, 64), thus discounting the possibility of this discrepancy being due to the membrane probes that we employed. Both the Glebov and Nichols study and ours quantitated probe clustering based on the effect of acceptor concentration on $E\%$. This approach requires maintaining a constant donor/acceptor ratio throughout the experiment, and insufficient monitoring of this ratio will affect interpretations regarding donor-acceptor co-clustering. We monitored this ratio using the corrected intensities of CFP and YFP (see “Experimental Procedures”), and we previously validated our corrections using CFP-YFP fusion proteins with established FRET properties (22). Importantly, our measurements were performed using ISs formed using T cells stimulated with pulsed B cells. In contrast, Glebov and Nichols (33) measured membrane caps in Jurkat cells stimulated using anti-CD3-coated latex beads. Thus, one possibility is that the caps have a nanarchitecture that is distinct from that of bona fide ISs. Furthermore, T cells often engulf beads used for stimulation (26, 33).

measurement of apparent plasma membrane in bead-stimulated T cells may therefore include a mixture of membranes from other compartments with distinct associative properties for membrane proteins.

The dynamic nature and small size of many rafts have kept their physical and biological properties elusive. Application of techniques such as FRET that provide information regarding the nanoscopic structure of cell membranes has driven an evolution in the interpretation of rafts. For example, although rafts were once envisioned as lipid scaffolds with which specific proteins associate (17), recent studies suggest that they form through complex interactions between proteins and lipids (65, 66). We present evidence here that actin filaments associated with the plasma membrane are one such set of proteins that structure rafts in the IS. Although earlier FRET studies failed to detect clustering of raft-associated proteins in the plasma membrane (67), including one measuring co-clustering in the IS (33), our data are consistent with studies that showed specific, cholesterol-dependent clustering of raft proteins and lipids in the plasma membrane (21–24, 40, 68, 69). One recent study resolved nanoscopic clustering of raft lipids by super-resolution imaging (24). As methods and instrumentation improve the resolution of membrane structure, existing discrepancies regarding the physical properties of raft proteins and lipids are likely to diminish.

Rearrangement of the T cell actin cytoskeleton following TCR engagement culminates with the formation of an IS. We show here that actin filaments in the IS are necessary to maintain rafts that regulate Lck in the IS. Actin polymerization and remodeling of the actin cytoskeleton are among the earliest events in cell activation, suggesting that signal regulation by actin-dependent rafts is a shared mechanism for controlling cell stimulation.

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