Phosphatase CD45 Both Positively and Negatively Regulates T Cell Receptor Phosphorylation in Reconstituted Membrane Protein Clusters**

Received for publication, April 16, 2014, and in revised form, August 5, 2014 Published, JBC Papers in Press, August 15, 2014, DOI 10.1074/jbc.M114.574319

Gabriela Furlan, Takashi Minowa, Nobutaka Hanagata, Chiho Kataoka-Hamai, and Yoshihisa Kaizuka

From the International Center for Materials Nanoarchitectonics, National Institute for Materials Science, Tsukuba, Ibaraki 305-0047, Japan

The roles of protein clustering in T cell signaling are poorly understood. Lck clustering recreates conditions in which a phosphatase has positive and negative roles in signal initiation. Autoinhibition due to Lck clustering is relieved by an optimal concentration of CD45. A novel regulatory mechanism of protein clustering other than increasing local concentration has been discovered.

T cell receptor (TCR) phosphorylation requires the kinase Lck and phosphatase CD45. CD45 activates Lck by dephosphorylating an inhibitory tyrosine of Lck to relieve autoinhibition. However, CD45 also dephosphorylates the TCR, and the spatial exclusion of CD45 from TCR clustering in the plasma membrane appears to attenuate this negative effect of CD45. To further investigate the role of CD45 in signal initiation, we reconstituted membrane TCR clusters in vitro on supported lipid bilayers. Fluorescence microscopy of single clusters showed that incorporation of CD45 enhanced phosphorylation of TCR clusters, but only when Lck co-clustered with TCR. We found that clustered Lck autophosphorylated the inhibitory tyrosine and thus could be activated by CD45, whereas diffusive Lck molecules did not. In the TCR-Lck clusters and at low CD45 density, we speculate that the effect of Lck activation may overcome dephosphorylation of TCR, resulting in a net positive regulation. The CD45 density in physiological TCR clusters is also low because of the exclusion of CD45. Thus, we propose that the spatial organization of TCR/Lck/CD45 in T cell membranes is important not only for modulating the negative role of CD45 but also for creating conditions in which CD45 has a positive role in signal initiation.

** This work was supported by a grant from the Sumitomo foundation (to Y. K.).
* This article was selected as a Paper of the Week.

The interplay of multiple kinases and phosphatases regulates various signaling pathways. For instance, T cell immune activation is a well studied system that involves such kinase-phosphatase networks (1). Ligation of the T cell receptor (TCR)2 with major histocompatibility complex peptide induces the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the TCR/CD3 complex by Lck, a membrane-anchored Src family tyrosine kinase (SFK). TCR phosphorylation by Lck leads to the formation of protein signaling complexes and subsequent downstream signaling (2). TCR phosphorylation and subsequent signaling also require a highly expressed transmembrane phosphatase, CD45 (3–5). However, TCR is also a substrate of CD45 (6). Thus, CD45 has dual positive and negative roles in the induction of TCR phosphorylation (7).

Lck, CD45, and TCR coexist and interact with each other in the plasma membrane of resting T cells, without inducing signaling. When TCR is bound to its ligand, TCR phosphorylation is induced by multiple regulatory mechanisms. First, Lck catalytic activity is regulated through the phosphorylation and dephosphorylation of two internal tyrosine residues that are conserved in SFKs (1). Phosphorylation of Tyr394, which is located in the activation loop of the kinase domain, results in catalytic activation. Conversely, phosphorylation of the other tyrosine, Tyr505 at the C terminus, is inhibitory because the intramolecular interaction between phosphorylated Tyr505 and internal SH2 domains results in autoinhibition. CD45 dephosphorylates Tyr(P)505 and increases the catalytic activity of Lck, and Lck activation by CD45 is thought to be indispensable for T cell signaling and development (1).

Another regulatory mechanism is clustering of TCR and other proteins in the plasma membrane. In recent imaging studies, protein clusters in submicron/micron sizes have been resolved in T cell plasma membranes, whereas the driving forces of the clustering have not been fully elucidated (8–12). These clusters are enriched with phosphorylated TCR, suggesting the clusters facilitate phosphorylation signaling. It is notable that these clusters spatially exclude CD45, probably because of steric hindrance of the CD45 major histocompatibility complex peptide induc
extracellular domain (9, 11). Thus, CD45 phosphatase activity is decreased and the balance of biochemical reactions is altered in the clusters (13–15).

These multiple regulatory mechanisms are coupled, resulting in diverse phenomena observed in T cell signaling. CD45-deficient mice and CD45 negative T cell lines show clear defects in T cell signaling induction, including defective positive selection in CD45-deficient mice (3–5). The phenotypes are rescued with only partial (<10%) reconstitution of CD45 (16, 17). However, titration studies demonstrate that CD45 expression levels correlate with the level of Lck phosphorylation, but not with that of TCR (16, 17). Biochemical studies show that Lck molecules purified from CD45 negative cells are hyperphosphorylated at both Tyr394 and Tyr505 and are highly active enzymes (18), and that resting T cells contain constitutively active Lck fractions (19). Meanwhile, the spatial exclusion of CD45 from TCR clusters in plasma membranes likely play a role in reducing TCR dephosphorylation, highlighting the negative regulatory role of CD45 in T cell signaling (13–15).

To dissect and quantitatively analyze TCR signaling, we developed biochemical approaches to reconstitute the system by creating clusters of recombinant signaling proteins on planar lipid bilayers to mimic protein clustering in T cell membranes. The planar geometry of the experimental system enables single cluster level analysis by fluorescence microscopy. In this system, we modulate the molecular composition, density, and two-dimensional molecular mobility in the clusters, and analyze how various parameters regulate signaling. We found that Lck clustering modulates the catalytic activity of Lck and the net outcome of TCR phosphorylation. Our results suggest a role for protein clustering, besides simply increasing the local concentration in the context of diffusion-limited reactions. We therefore investigated how such effects might underlie the dual positive/negative regulatory functions of CD45 in TCR phosphorylation.

**EXPERIMENTAL PROCEDURES**

*Materials*—1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS), 1,2-dipalmityl-sn-glycero-3-phosphatidyl ethanolamine-N’-(cap biotinyl) (biotin-cap-PE), and 1,2-dioleoyl-sn-glycerol-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl] (DGS-NTA(Ni)) were obtained from Avanti Polar Lipids (Alabaster, AL). 1,1’-Diocadecyl-3,3,3’,3’-tetrathymethylindocarbo-cyanine perchlorate (DiD) and tetramethylrhodamine thioacetamido-1,2-dihexadecanoyl-sn-glycero-2-phosphoethanolamine, triethylammonium salt (TRITC-DHPE) were obtained from Invitrogen, and perylene was obtained from Sigma. Cy3-streptavidin, Alexa 546-C5-maleimide, Alexa 647-C2-maleimide, and Alexa 488 TFP (Alexa 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester) were obtained from Invitrogen. Antibodies were then added to pre-cluster lipids in supported bilayers (for both syn-protein clusters and His10-protein clusters). Before adding proteins, the bilayer samples were washed with buffer (30 mM HEPES-NaOH (pH 7.4)) and 5 mM MgCl2 was then added to pre-cluster lipids in supported bilayers (for both syn-protein clusters and His10-protein clusters). Before adding proteins, the bilayer samples were washed with buffer (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl). By including Mg2+ prior to protein conjugation, we prevented or minimized the formation of new protein clusters at later time points when Mg2+ was necessary for phosphorylation. Note that once formed, these lipid/protein clusters on supported bilayers were stable during the experimental procedures (data not shown).

To create signal protein clusters, bilayers were formed under buffer (30 mM HEPES-NaOH (pH 7.4)) and 5 mM MgCl2 was then added to pre-cluster lipids in supported bilayers (for both syn-protein clusters and His10-protein clusters). Before adding proteins, the bilayer samples were washed with buffer (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl). By including Mg2+ prior to protein conjugation, we prevented or minimized the formation of new protein clusters at later time points when Mg2+ was necessary for phosphorylation. Note that once formed, these lipid/protein clusters on supported bilayers were stable during the experimental procedures (data not shown). For the experiments without protein clusters, supported bilayers were created under buffer without divalent cations (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl).

Proteins were then added for conjugation to the bilayers, followed by blocking with 0.1 mg/ml of BSA. 5 mM ATP, 1 mM MgCl2 was added to trigger phosphorylation. For real-time detection of phosphorylation, 100 nM Alexa 488-labeled anti-pY142-CD3ζ antibody or 100 nM AcGFP-tSH2 was included in the ATP/Mg mixture, whereas for kinetic and steady-state analysis, probes were added after the reactions.

In Vitro Reconstitution of T Cell Signaling Protein Clusters

Recombinant Protein Expression, Purification, and Labeling—Lck proteins were expressed in S9 cells using a bac-to-bac system (Invitrogen). Full-length human Lck and Y505F mutant genes conjugated to His10 or α-synuclein (full-length) at the N terminus were cloned into the pFastBac1 vector containing the GST gene and PreScission protease (GE Healthcare) cleavage site at the cloning site, and proteins were expressed in S9 cells and purified using glutathione-Sepharose 4B (GE), PreScission Protease, and gel filtration. The cytoplasmic domain of human CD3ζ (amino acids 53–163) fused to His10 or α-synuclein at the N terminus, cytoplasmic domain of human CD45 (amino acids 584–1281) fused to His10 at the N terminus and fluorescent proteins (TagBF or mCherry) at the C terminus, and tandem SH2 domain of ZAP70 gene (amino acids 1–259) fused with AcGFP (Aequorea coerulescens green fluorescent protein) at the C terminus were cloned into the pGEX6p-1 vector, expressed in BL21 Escherichia coli, and purified using glutathione-Sepharose 4B (GE Healthcare), PreScission Protease, and gel filtration. CD3ζ and Lck were labeled using maleimide-conjugate Alexa 647 and Alexa 546, respectively, at an artificially introduced cysteine (CD3ζ) or at surface cysteines (Lck). Antibodies were labeled using TFP (tetrafluorophenyl ester)-conjugated dyes (Alexa 488). The concentrations and labeling efficiency of the purified proteins were measured using a spectrophotometer.

Reconstitution of Signaling Protein Clusters and Reactions on Supported Lipid Bilayers—Liposomes were prepared by extrusion through a 100-nm polycarbonate filter and deposited on glass coverslips cleaned by piranha solution (a mixture of sulfuric acid and hydrogen peroxide) to form a single fluid planar bilayer. Lipid compositions of the supported bilayers were: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC for the syn-protein systems, and the concentration range of fluorescent molecules (0.02–0.6% perylene, 0.0025–0.05% TRITC-DHPE, and 0.02–0.25% DiD) and DOPC for the fluorescence calibration standards.

To create signal protein clusters, bilayers were formed under buffer (30 mM HEPES-NaOH (pH 7.4)) and 5 mM MgCl2 was then added to pre-cluster lipids in supported bilayers (for both syn-protein clusters and His10-protein clusters). Before adding proteins, the bilayer samples were washed with buffer (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl). By including Mg2+ prior to protein conjugation, we prevented or minimized the formation of new protein clusters at later time points when Mg2+ was necessary for phosphorylation. Note that once formed, these lipid/protein clusters on supported bilayers were stable during the experimental procedures (data not shown). For the experiments without protein clusters, supported bilayers were created under buffer without divalent cations (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl).

Proteins were then added for conjugation to the bilayers, followed by blocking with 0.1 mg/ml of BSA. 5 mM ATP, 1 mM MgCl2 was added to trigger phosphorylation. For real-time detection of phosphorylation, 100 nM Alexa 488-labeled anti-pY142-CD3ζ antibody or 100 nM AcGFP-tSH2 was included in the ATP/Mg mixture, whereas for kinetic and steady-state analysis, probes were added after the reactions.
Protein clusters in the digital images were detected semi-automatically using ImageJ (National Institutes of Health, Bethesda, MD). Pixels with intensity above the calculated threshold level were separated from background to produce binary images. Clusters containing either CD3ζ or Lck yielded high contrast fluorescent images, and the detection was straightforward and reproducible. These binary images were applied to the original image data and the information of each cluster (size and average fluorescence intensities of all channels) was obtained.

We examined the spectral overlap by imaging each fluorescent protein alone in all four channels. To correct the spectral overlap, the following adjustments were performed prior to protein density calibrations. 1) To remove the weak emission of Alexa 546-tagged Lck in the green filter (excitation 475/40, emission 530/50 nm), green channel fluorescent signals in the absence of green fluorescent probes (Alexa 488 antibodies and AcGFP-tSH2) were recorded before initiating reactions and considered as background intensities. Background intensities were subtracted after staining with green fluorescent probes. 2) Green probe emission was detected in the blue channel (excitation 405/20, emission 460/50 nm). Thus, blue channel signals without TagBFP-CD45, with or without green probes, were obtained. TagBFP signals in the blue channel were then corrected by subtracting both background and channel bleed signals. 3) Spectral overlap of dyes between the red (excitation 555/25, emission 620/60 nm) and the near infrared (excitation 620/60, emission 700/75 nm) filters may result in the overestimation of protein densities, which were ignored in the analysis (errors were at most 0.019 Lck molecules/μm² per one Alexa 647-CD3ζ molecule/μm² and 0.0075 CD3ζ molecules/μm² per one Alexa 546-Lck molecule/μm²). Other spectral overlaps were not detected in our experimental conditions.

Fluorescence Recovery after Photobleaching (FRAP)—FRAP assays were performed using a Nikon (Tokyo, Japan) Eclipse Ti-E microscope equipped with an iXonEM EMCCD camera (Andor, Belfast, UK). Experiments were performed by time lapse imaging after short photobleaching (<1 s) with laser at 561 (Sapphire LP, 200 milliwatt, Coherent, Santa Clara, CA) and 640 nm (561CS/S2669, CVI Laser Optics and Melles Griot, Albuquerque, NM). The mobile fraction in homogeneous membranes was determined by the ratio between the initial fluorescence and the maximum fluorescence 5 min after photobleaching. The diffusion coefficient (D) was estimated using the following equation: 

\[ D = 0.22w^2/\tau_{0.5} \] 

(\( w \) = the bleach spot radius), derived for the FRAP curve in brief and uniform circular bleaching spots in homogeneous two-dimensional lipid membranes (21). For the FRAP curves of molecules in clusters that exhibited anomalous diffusion, the effective diffusion coefficient (D_{eff}) was obtained through the fit of the initial period of anomalous FRAP curves with an exponential (the equation for normal two-dimensional diffusion). Mobile fractions for slowly recovering molecules were calculated from the maximum fluorescence within 5 min of the post-photobleach, even when the bleached spots may not be fully recovered because of slow diffusion.

Imaging of Reactions in the Reconstituted Systems—Samples were imaged by the Leica (Solms, Germany) AF6000LX total internal reflectance (TIRF) microscopy equipped with a 100 × 1.46 NA oil-immersion objective and a Cascade II EMCCD camera (Roper, Tucson, AZ). Antibodies (Alexa 488-labeled) and AcGFP-tagged tandem SH2 domain of ZAP70 were imaged by objective-based TIRF microscopy using a 488-nm solid-state diode laser (20 milliwatt). Fluorescence intensities of labeled Lck, CD45, and CD3ζ were measured by epi-fluorescence imaging after washing away excess protein with wash buffer (30 mM HEPES-NaOH, pH 7.4, and 150 mM KCl).

Kinetic parameters were obtained from reactions stopped at 30 s, when the phosphorylation increased in the initial rate even at high Lck density (−300 μm⁻²) (Fig. 1C). The reaction was stopped with wash buffer, and images obtained after supported bilayers were incubated with probes for 5 min and washed. To examine steady-state network reactions, images were obtained from proteins reacted for 8 min, incubated with probes for 5 min, and washed.

Near steady-state levels of Lck Tyr(P)³⁹⁴ and Tyr(P)⁵⁰⁵ were measured in a similar manner, but with 100 nm Alexa 488-labeled anti-Tyr(P)-antibodies (pY505-Lck and pY416 c-Src). Images were obtained after reacting for 8 min, incubating for 5 min with antibodies, and washing with buffer. CD3ζ cluster density in these assays was ~1000 μm⁻².

Image Analysis—To quantify protein densities, fluorescent protein clusters were calibrated with fluorescent standards, as described previously (20). Standards were created from the images of bilayers containing fluorescent molecules (0.02–0.6% perylene, 0.0025–0.05% TRITC-DHPE, and 0.02–0.25% DiD), in the blue (excitation 405/20, emission 460/50 nm), red (excitation 555/25, emission 620/60 nm), and near infrared (excitation 620/60, emission 700/75 nm) channels, respectively. Fluorescence intensities increased linearly in this concentration range, which covered the protein concentrations used in the experiments. To directly compare protein images with membrane standards, protein and membrane dye fluorescence was compared by fluorimetry (F-7000 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan). Protein and dye emission spectra in buffer were obtained at excitation and emission wavelengths of the microscopy filters to establish a baseline. Integrated fluorescence intensities were calculated from two-dimensional spectra and the arc lamp spectrum of microscope. Ratios of the integrated intensities between proteins and membrane standard dyes were used as the scaling factors in the calibration (20).

Fluorescence of Alexa 488-antibodies was calibrated relative to the fluorescence of antibodies bound to nearly fully phosphorylated CD3ζ on supported bilayers (30 min reaction). We assumed 100% phosphorylation in prephosphorylated CD3ζ, which may result in a slight overestimation in the calculated pCD3ζ density and k_{o}. For steady-state analysis, the amount of Alexa 488-antibodies and AcGFP-tSH2 bound to pY142-CD3ζ on supported bilayers were normalized in each experimental set, but were not calibrated in a single scale. This was to minimize the errors in TIRF illuminations between different experiments.
Cell Imaging—Jurkat cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS). Supported bilayers (0.1% biotin-cap-PE lipid and DOPC) were created on etched coverslips (by piranha solution) under saline HEPES buffer (20 mM HEPES-NaOH, pH 7.4, 135 mM NaCl, 4 mM KCl, 1 mM Na2HPO4, 10 mM glucose, 1 mM CaCl2, 0.5 mM MgCl2). Jurkat cells were stimulated on supported bilayers that displayed anti-TCR antibody that were biotinylated and then conjugated to biotin-cap-PE in the bilayers through Cy3-labeled streptavidin as previously described (22). After 5 min of stimulation, the cells on bilayers were fixed with 3% paraformaldehyde (15 min) and permeabilized with 0.1% Triton X-100 (2 min). Those cells were blocked with 0.1% BSA and stained with 100 nM labeled antibodies (Alexa 488-labeled anti-pY142-CD3ζ, pY394-Lck, and pY416 c-Src) that were also used in the *in vitro* experiments. Stained cells were imaged by TIRF microscopy using the same protocol as for the imaging of reconstituted protein clusters.

**RESULTS**

**Imaging of Reconstituted T Cell Signaling in Vitro on Planar Lipid Bilayers**—We expressed and purified recombinant proteins, including the cytoplasmic domains of Lck, CD45, and CD3ζ (a part of the TCR/CD3 complex that contains three ITAMs) to biochemically reconstitute T cell signaling in vitro. These proteins form a signaling network that facilitates the phosphorylation/dephosphorylation of tyrosine residues in ITAMs and Lck (Tyr394 and Tyr505) (Fig. 1A). Lck autophosphorylation modulates Lck catalytic activity (Fig. 1B). These proteins were fused with His10 at their N terminus and anchored to DGS-NTA(Ni) lipids in fluid supported bilayers. Thus, the signaling network was reconstituted on a membranous surface as in T cells, labeled with different dyes, and imaged by fluorescence microscopy. All proteins uniformly bound to the bilayers and were diffusive, which was confirmed by FRAP analysis (Fig. 1C).

To monitor Lck phosphorylation of CD3ζ on supported bilayers, we triggered the reaction with ATP and imaged the binding of an Alexa 488-labeled antibody specific to phosphorylated Tyr412 in the ITAM of CD3ζ. TIRF imaging of antibody binding enabled real time measurement of ITAM phosphorylation (Fig. 1D). Bilayer protein density was measured by calibrating protein fluorescence intensities as previously reported (20). Within the range of physiological densities of Lck and CD3ζ (300 μm−2 for both proteins (23)), we observed that CD3ζ phosphorylation proceeds to 50% within 30 s and is close to saturation in 5 min in the absence of CD45 (Fig. 1D). This time scale is comparable with that reported recently on liposomes (24). Note that there could be a delay in the detection of phosphorylation because antibody binding is not instantaneous, and antibody binding to pre-phosphorylated CD3ζ on supported bilayers was nearly 90% complete within 30 s (Fig. 1D). We confirmed that antibody binding was at undetectable levels when the system lacked either Lck or CD3ζ (data not shown).

To determine the two-dimensional kinetic parameters of Lck on bilayers, a plot of the initial rates of CD3ζ phosphorylation at low Lck density (1.44 μm−2) was obtained and fit using the Hill equation (Fig. 1E). We observed a positive allosteric effect (Hill coefficient 4.23), as reported previously (24). The *k* cat measured in our system (0.21 s−1) is orders of magnitude higher than the value obtained in solution phase but is smaller than that obtained on liposomes (Table 1) (24–26). The classical kinetic model used for the fitting did not incorporate the effects of two-dimensionality and lower molecular mobility on supported bilayers relative to the mobility in solution or in support-free liposomes. Such effects may in part underlie differences in the observed rate constants (27, 28).

We also found that at even lower Lck densities (below the detection limit), the initial phosphorylation rates followed basic Michaelis-Menten kinetics (Hill coefficient 1.1) (Fig. 1F). Thus, allosteric effects at higher Lck density may be related to Lck autophosphorylation, which could mediate the allosteric interactions between Lck and ITAMs.

**Reconstitution of Signal Protein Clusters**—To investigate how protein clustering regulates signaling in T cell membranes, we sought to reconstitute protein clusters on lipid bilayers. For the cluster formation, we used lipid and protein self-assembly. Lipids in bilayers self-assemble into various macroscopic structures, such as liquid-disordered, liquid-ordered, and gel phases, and various proteins are reported to self-assemble on lipid membranes or associate with those lipid phases (29, 30). Thus, we examined combinations of proteins and lipids to create clustering structures on supported membranes that mimic the spatial organization of T cell membranes. In particular, we tested different N-terminal tags for proteins. Signaling proteins in T cell membrane clusters, including Lck, exhibit two distinct mobility states: diffusive and non-diffusive, depending on the T cell activation state (9, 31). We sought to model such fast and slow protein diffusion by introducing different N-terminal tags. One such tag was α-synuclein, a protein related to Parkinson’s disease. α-Synuclein was shown to self-assemble on supported bilayers that contain negatively charged lipids (e.g., phosphatidyglycerol or phosphatidylserine), and that clustering was enhanced by divalent cations (e.g., Ca2+ and Mg2+) (32). Alternatively, phase-separated domains of anionic lipids are induced by divalent cations alone (29, 30), and α-synuclein localizes to these lipid domains (32). These two approaches seem to form identical clusters (32). We also found that clusters created using combinations of either anionic lipids (phosphatidyglycerol or phosphatidylserine) or cations (Ca2+ or Mg2+) similar in morphology, protein density, and mobility (data not shown). These results suggest that the basis of α-synuclein clustering may be lipid phase separation, and both α-synuclein and cationic ions interact with, nucleate, and phase-separate anionic lipids (29, 30). The phase separation was more robust in low salt, suggesting modulation by monovalent ion concentration (32). Intermolecular interactions of α-synuclein related to amyloid formation are also likely involved (33).

CD3ζ and Lck that include α-synuclein (syn−) tags at the N terminus were prepared for clustering on supported bilayers. Although there are various ways to create very similar protein clusters as described above, we pre-clustered DOPS (25%) with Mg2+ (5 mM MgCl2) in the bilayers that also contained 5% DGS-NTA(Ni), and conjugated the proteins (syn-CD3ζ, syn-Lck, and His10-CD45). We observed that syn-CD3ζ and syn-Lck proteins co-clustered and partially segregated from His10-
FIGURE 1. Reconstitution of TCR-Lck-CD45 reaction network on supported lipid bilayers. A, schematic drawing of the reconstituted system and the reaction network. Purified recombinant proteins fused with His10 tags are attached to DGS-NTA(Ni) in supported lipid bilayers. Lck phosphorylates CD3ζ/H9256 as well as Lck (autophosphorylation), and CD45 dephosphorylates both CD3ζ and Lck. TIRF imaging of the binding of fluorescent probes (labeled antibody and ZAP70) to phospho-CD3ζ facilitates dynamic monitoring of CD3ζ phosphorylation. B, schematic drawing of Lck activity mediated by Tyr505 phosphorylation/dephosphorylation and the formation/release of autoinhibitory structure. C, FRAP analysis of His10-proteins (Alexa 647-His10-CD3ζ, Alexa 546-His10-Lck, and His10-CD45-mCherry) on supported bilayers (5% DGS-NTA(Ni) and 95% DOPC). Representative images of the photobleached spots (pre-bleach images and post-bleach images (t = 0 s, 5 min)) are shown (left). From the plots of normalized intensities in the photobleached spots (right), parameters (mobile fraction and diffusion coefficient) were obtained. Diffusion coefficients were 0.42 m2/s (His10-CD3ζ), 0.64 m2/s (His10-Lck), and 0.54 m2/s (His10-CD45). Scale bar = 10 μm. D, monitoring Lck-catalyzed phosphorylation of CD3ζ. Time-lapse TIRF images of Alexa 488-labeled anti-pY142-CD3ζ antibody binding to phosphorylated CD3ζ on supported bilayers (left) and the normalized fluorescence intensities of those images (solid line, right). Protein densities of CD3ζ and Lck: ~300 μm⁻². Lipid composition of supported bilayers: 5% DGS-NTA(Ni) and 95% DOPC. Time course of normalized fluorescence intensities of the labeled antibody bound to pre-phosphorylated CD3ζ on supported bilayers was also plotted (dashed line). Pre-phosphorylation was performed on supported bilayers for 8 min (densities of CD3ζ and Lck: ~300 μm⁻²). Scale bar = 5 μm. E and F, kinetic analysis of CD3ζ phosphorylation on supported bilayers at an average Lck density = 1.44 μm⁻² (E) or lower (undetectable by fluorescence) (F). Data were fitted with an allosteric sigmoidal curve and two-dimensional kinetic parameters (kcat = 0.21 s⁻¹, KM = 203.7 μM⁻² (E) or 86.5 μM⁻² (F) and Hill constant, nH = 4.23 (E) or 1.1 (F)) were obtained, respectively. Lck density in F could not be measured, because fluorescence intensity could not be detected. Thus, we could not obtain kcat and the catalytic activity (kcat/KM) for the data in F. Error bars represent S.E. pY142-CD3ζ, phosphorylated Tyr142 in CD3ζ.
CD45 as observed in T cell membranes (Fig. 2A, note that CD45 density is lower in the clusters), suggesting that DGS-NTA(Ni) was partially excluded from the clusters. Protein densities in individual clusters were determined by calibrating the fluorescence intensities as described previously (Fig. 2A) (20).

FRAP analysis indicated that syn-Lck was immobile in the clusters. In contrast, syn-CD3ζ and His10-CD45 contained substantial mobile fractions (26.1 and 39.1%, respectively), indicating a rapid molecular exchange between the inside and outside of the clusters (Fig. 2B). Although the molecular mobility is significantly altered in syn-Lck-enriched clusters, CD3ζ was still robustly phosphorylated (Fig. 2C). Syn-Lck molecules outside the clusters were not completely immobilized (mobile fraction = 15%, D = 0.038 μm²/s, data not shown). FRAP curves in Fig. 2B suggest that syn-CD3ζ and His10-CD45 diffusion was anomalous, probably because both slow molecules in clusters and fast molecules out of clusters were mixed by diffusion. Effective diffusion coefficients for anomalous diffusion (D_{eff} = 0.21 μm²/s (syn-CD3ζ) and 0.20 μm²/s (His10-CD45)) were obtained by fitting the initial periods of the FRAP curves in Fig. 2B with the exponential curve.

We speculate that syn-CD3ζ was loosely co-aggregated with syn-Lck and lipids, and His10-CD45 was anchored to fluid DGS-NTA(Ni) lipids, and thus percolate or diffuse much faster in those systems, either in homogeneous membranes or in clusters (Fig. 3A and B). At physiological Lck density (~300 μm⁻²), very strong CD3ζ phosphorylation was observed in the absence of CD45. However, CD3ζ phosphorylation levels were sharply decreased in the presence of only 50–100 μm⁻² CD45 molecules, which is significantly lower than the physiological CD45 density in T cell membranes (~1000 μm⁻²) (34). This trend is in good agreement with previous measurements in solution phase or on liposomes (6, 24). CD3ζ phosphorylation in the absence of CD45 appeared to saturate at lower Lck density (~100 μm⁻²) in homogeneous membranes, whereas that in His10-protein clusters increased with Lck density (100–400 μm⁻²), suggesting a slower reaction rate in the clusters (Fig. 3C).

In contrast, we obtained bell-shaped curves for CD3ζ phosphorylation in syn-protein clusters. We consistently observed that low CD45 densities (100–200 μm⁻²) enhanced CD3ζ phosphorylation levels (1.5–2.5-fold) at intermediate Lck densities (400–600 μm⁻²) (Fig. 3D). Meanwhile, CD3ζ phosphorylation decreased at higher CD45 densities, indicating the CD3ζ dephosphorylation by CD45 was active in this system. Near CD45 physiological density (~1000 μm⁻²), the syn-CD3ζ phosphorylation level seemed to be dominated by the phosphatase activity of CD45, resulting in reduced phosphorylation.

To investigate whether enhanced phosphorylation in syn-CD3ζ by CD45 was due to syn-Lck activation through

### In Vitro Reconstitution of T Cell Signaling Protein Clusters

#### Table 1

Two-dimensional kinetic parameters of Lck on membranes

<table>
<thead>
<tr>
<th>Membrane system</th>
<th>( n_{tr} )</th>
<th>( k_{cat} ) (s⁻¹)</th>
<th>( K_m ) (μm⁻²)</th>
<th>( k_{cat}/K_m ) (×10⁻⁶ μm²)</th>
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<tr>
<td>Supported bilayer</td>
<td>4.2</td>
<td>0.21</td>
<td>204</td>
<td>1.03</td>
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<tr>
<td>Liposome (24)</td>
<td>2.3</td>
<td>3.41</td>
<td>245</td>
<td>1.39</td>
</tr>
<tr>
<td>In solution (25)</td>
<td>0.0002–0.0098</td>
<td>2.3–21.7 (×10⁻⁵ m)</td>
<td>1.8–121.8 (m⁻¹ s⁻¹)</td>
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dephosphorylation of the inhibitory tyrosine 505 (Fig. 1B), we examined mutant syn-Lck Y505F that lacked Tyr505 phosphorylation. As expected, enhanced CD3ζ phosphorylation at low CD45 density was not observed in clusters containing syn-Lck-Y505F (Fig. 3E), indicating a causal relationship between the stimulatory function of CD45 in syn-CD3ζ phosphorylation and Tyr505 dephosphorylation. syn-Lck net kinase activity increased with Lck density and the activity of Lck-Y505F, which lacks the autoinhibitory interaction, was stronger than that of wild-type Lck, as expected (Fig. 3F).

We analyzed reactions in individual clusters without any boundary conditions, ignoring the effects of two-dimensional...
finite volume. Accordingly, reactions in individual clusters were assumed independent of molecules outside the clusters. To assess the accuracy of these assumptions, we analyzed the effect of cluster size and found that CD3ζ phosphorylation levels were independent of cluster size (Fig. 3G). This suggests that the effect of boundary reactions is rather minor and supports the assumption of independent reactions within individual clusters. We also found that CD3ζ phosphorylation levels were not dependent of CD3ζ density, remaining largely unchanged at CD3ζ densities of 500–1200 μm⁻² (data not shown), similar to physiological TCR cluster density (20).

We also examined whether in vitro syn-protein clusters could recruit ZAP70. ZAP70 is a direct downstream signaling protein that binds to phosphorylated ITAMs and localizes in TCR clusters in activated T cells (12). We incorporated the GFP-tagged ITAM binding domain of ZAP70 in a reconstituted system and found significant levels of ZAP70 binding to syn-protein clusters at greater than or similar to physiological Lck density (~300 μm⁻²) (Fig. 3, H and I), suggesting that CD3ζ phosphorylation levels in syn-protein clusters are sufficient to induce downstream signal transduction.

Lck Autophosphorylation in Clusters—To further investigate how Lck activity is regulated in reconstituted clusters, we imaged and quantified Tyr³⁹⁴ and Tyr⁵⁰⁵ phosphorylation levels by staining with two phosphotyrosine-specific antibodies. We observed substantial Tyr⁵⁰⁵ phosphorylation of syn-Lck (Fig. 4, A and B). As Lck was the only kinase in the system, Tyr⁵⁰⁵ was phosphorylated through either intermolecular or intramolecular autophosphorylation. Tyr³⁹⁴ phosphorylation in syn-Lck clusters decreased monotonically with increasing CD45 density, but was preserved at high CD45 densities, indicating rapid autophosphorylation of Tyr³⁹⁴ in syn-Lck clusters that competes with dephosphorylation by CD45 (Fig. 4, A and B). Conversely, Tyr⁵⁰⁵ phosphorylation in diffuse His₁₀-Lck clusters was much less, even in the absence of CD45, and decreased further in the presence of low levels of CD45 (Fig. 4, A and B). The specificity of the Tyr(P)⁵⁰⁵ antibody over pY142-CD3ζ and Tyr(P)³⁹⁴ was evaluated using Lck-Y505F mutants (data not shown).

Phosphorylation of Tyr³⁹⁴ enhances Lck catalytic activity (1), and we observed robust Tyr³⁹⁴ phosphorylation in both syn-Lck and His₁₀-Lck clusters (Fig. 4, C and D). Thus, the phosphorylation ratio of tyrosine residues (Tyr(P)³⁹⁴/Tyr(P)⁵⁰⁵) in His₁₀-Lck clusters was greater than that in syn-Lck clusters over a wide range of CD45 densities. In this situation, the catalytic activity of His₁₀-Lck was likely governed by the dephosphorylation of Tyr³⁹⁴ rather than that of Tyr⁵⁰⁵, suggesting the activity was negatively regulated by CD45. Accordingly, CD3ζ phosphorylation in the His₁₀-Lck protein system was not enhanced by CD45. Conversely, the level of Tyr³⁹⁴ phosphorylation in syn-Lck was substantial but also unchanged over a wide range of CD45 densities (Fig. 4D). Therefore, syn-Lck cluster catalytic activity may be governed primarily by the phosphorylation/dephosphorylation of Tyr⁵⁰⁵.

Phosphorylation and Signal Transduction in T Cells—To investigate whether regulatory mechanisms observed in the reconstituted systems could contribute to physiological TCR signaling, we analyzed TCR phosphorylation signals in membrane clusters of Jurkat T cells. Jurkat cells formed signaling protein clusters by interacting with stimulatory anti-TCR antibodies displayed on lipid bilayers, as in primary T cells (Fig. 4E) (22). The CD3ζ phosphorylation occurred exclusively in TCR clusters (22), as shown by immunofluorescence staining with the labeled anti-pY142-CD3ζ antibody that was used in the reconstituted systems (Fig. 4E).

We detected phosphorylation of Lck in TCR clusters of Jurkat T cell membranes by immunofluorescence staining with the same two antibodies used in the reconstituted systems. Robust Tyr(P)³⁹⁴ and Tyr(P)⁵⁰⁵ signals were observed in the TCR clusters (Fig. 4, E and F), similar to those seen in syn-protein clusters (Fig. 4, A and C). Although it is not straightforward to quantitatively compare the results in cells and in reconstituted clusters, Tyr⁵⁰⁵ phosphorylation in T cell membranes is likely much higher than that for His₁₀-Lck in diffusive clusters and was localized in the TCR clusters.

**DISCUSSION**

Our studies suggest that Lck Tyr⁵⁰⁵ is highly autophosphorylated in protein clusters when Lck is immobile. Additionally, Tyr(P)⁵⁰⁵ levels in immobilized Lck were sustained over a wide range of CD45 densities, indicating autophosphorylation activity that competes with dephosphorylation by CD45. Conversely, autophosphorylation on Tyr³⁹⁴, a positive regulatory site, was observed under all conditions tested, and may play a key role in regulating catalytic activity of diffuse Lck that lacks Tyr⁵⁰⁵ autophosphorylation.

This observation is largely consistent with previous analyses of c-Src and Lck autophosphorylation (24, 35). Autophosphorylation of both SFKs predominantly occurs at Tyr³⁹⁴ (Lck) and Tyr⁴¹⁶ (c-Src, corresponding to Lck Tyr³⁹⁴) through intermolecular reactions, whereas a C-terminal inhibitory tyrosine (Tyr⁵⁰⁵ in Lck and Tyr⁵²⁷ in c-Src) is autophosphorylated both intra- and intermolecularly. However, the autophosphorylation of Tyr⁵⁰⁵/Tyr⁵²⁷ has a much higher Kₘ for ATP than Tyr³⁹⁴/Tyr⁴¹⁶.
Tyr^{416} phosphorylation (24, 35). Thus, when intermolecular interactions occur, Tyr^{394}/Tyr^{416} is the dominant autophosphorylation site. However, once Lck clusters and is immobilized, intermolecular interactions may be less favorable, and Tyr^{505}/Tyr^{527} cis-autophosphorylation through intramolecular interactions might increase. In solution, dilution of SFKs increases intramolecular cis-autophosphorylation of the inhibitory tyrosine, instead of immobilization (35).

Based on our analysis, we propose a model that explains our observations in the context of the molecular spatial organization, Lck mobility and activity, and net TCR phosphorylation (Fig. 5, A and B). In syn-protein clusters, immobile Lck autophosphorylates Tyr^{505}, and CD45 dephosphorylates Tyr(P)^{505} to relieve the autoinhibition. At a reduced CD45 density, the effect of Tyr^{505} dephosphorylation and subsequent Lck activation overcomes the CD45 dephosphorylation of CD3ɛ/H9256, resulting in a net positive regulation of CD3ɛ/H9256 phosphorylation. Conversely, diffuse Lck autophosphorylates exclusively at Tyr^{394}, a positive regulatory site, and thus CD45 acts only negatively on both Lck and CD3ɛ. Therefore, the phosphorylation level of

FIGURE 3. Steady-state phosphorylation of CD3ɛ in the reaction network with Lck and CD45 in differentially reconstituted clusters and homogeneous membranes. The CD3ɛ phosphorylation level was plotted as the ratio to the total CD3ɛ density (pY142-CD3ɛ/CD3ɛ) at different Lck and CD45 densities. A and B, CD3ɛ phosphorylation levels in the network of His{tagged}-proteins in homogeneous membranes (A) and diffusive His{tagged}-proteins in clusters (B) were plotted following normalization to the phosphorylation levels of CD45 = 0 μm{sup}-2. Lipid compositions in supported bilayers: 5% DGS-NTA(Ni) and 95% DOPC. C, CD3ɛ phosphorylation levels in A and B at different Lck densities in the absence of CD45 (CD45 = 0 μm{sup}-2) compared and normalized in a single scale. D, CD3ɛ phosphorylation levels in the syn-protein clusters were plotted after normalization to the phosphorylation levels of CD45 = 0 μm{sup}-2. Lipid composition of supported bilayers: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC. E, normalized CD3ɛ phosphorylation levels in syn-protein clusters containing syn-Lck-Y505SF mutant proteins. Bilayer compositions are the same as in D. F, CD3ɛ phosphorylation levels in clusters containing either syn-Lck (wild-type) or syn-Lck-Y505SF at different Lck densities, in the absence of CD45 (CD45 = 0 μm{sup}-2, D and E), were compared and normalized in a single scale. G, phosphorylated CD3ɛ levels in syn-protein clusters that were further normalized with Lck densities plotted at different cluster sizes. H, detection of CD3ɛ phosphorylation in syn-protein clusters by the AcGFP-tagged tandem SH2 domain of ZAP70. Reconstituted protein clusters contained α-synuclein (syn-) tagged CD3ɛ (Alexa 647-labeled) and Alexa 546-labeled syn-Lck, and partially excluded His{tagged}-CD45-TagBFP. Lipid compositions in supported bilayers were: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC. I, detection of CD3ɛ phosphorylation in syn-protein clusters by the AcGFP-tagged tandem SH2 domain of ZAP70. Reconstituted protein clusters contained α-synuclein (syn-) tagged CD3ɛ (Alexa 647-labeled) and Alexa 546-labeled syn-Lck, and partially excluded His{tagged}-CD45-TagBFP. Lipid compositions in supported bilayers were: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC.
CD3ζ decreases monotonically with increasing CD45 density in the network that includes diffusive Lck.

Robust Tyr 505 and Tyr 394 phosphorylation were also observed in T cell membrane clusters, suggesting that a fraction of Lck in T cells may be immobilized in TCR clusters and regulated in a manner similar to immobilized syn-Lck in the in vitro clusters. Previous single molecule studies demonstrated a substantial Lck immobile fraction in T cell membranes, particularly after T cell activation (9, 31), because of trapping of Lck molecules in the clusters via protein-protein interactions (9). Lck interacts with phospho-ITAM (36, 37). Thus, the competition of the intermolecular and intramolecular interactions between phosphorylated tyrosine (ITAM and Tyr 505) and the SH2 domain may explain Tyr 505 phosphorylation and transient trapping of Lck in TCR clusters (Fig. 5C). Lck proteins in the two distinct reconstituted clusters may represent physiological Lck at two distinct mobility states, which may be modulated by interactions with other proteins in T cells. In our reconstitution studies, Lck mobility was regulated mostly by lipid-protein interaction or interactions between -synuclein molecules. Thus, development of a new clustering strategy is required to further investigate the effects of Lck trapping.

Additionally, super-resolution imaging revealed Lck self-clustering that depends on its conformational state (38), and FRET analysis detected conformational changes in a fraction of Lck after T cell activation (39). Moreover, it has been known that CD4/CD8-Lck interactions may contribute to the signaling (40). Collectively, these results suggest a fine-
tuning of Lck activity depending on its structure and clustering in T cells.

In T cell membranes, TCRs cluster and exclude CD45. This spatial organization has been thought to be important to attenuate the negative role of CD45: the dephosphorylation of TCR. In this spatial pattern, signal induction out of clusters, where TCR densities are low, is also prevented. However, our in vitro studies demonstrate that stimulation of TCR phosphorylation by CD45 also occurs at low CD45 density. Thus, we propose that the separation of TCR and CD45 may also be important for the positive role of CD45: a small amount of CD45 distributed in TCR clusters may positively regulate TCR phosphorylation.

Other factors are also involved in T cell activation. C-terminal Src kinase phosphorylates Lck Tyr505 (1), which is synergistic with Tyr505 cis-autophosphorylation. Embedding of CD3 proteins in the plasma membrane modulates the initial rate of ITAM phosphorylation (41). Actin polymerization is critical for protein clustering and signaling (11), and kinase ZAP70 and cytoplasmic phosphatases (e.g. SHP-1) also likely phosphorylate or dephosphorylate ITAMs (1). Thus, testing of these molecules in our reconstitution system may form the basis of future experiments. Throughout our analysis, the signal was evaluated at the level of pY142-CD3ζ per CD3ζ, as a measure for the phosphorylation activity of the molecular network. However, the recruitment of ZAP70 and downstream signaling mediated by ZAP70 may not be linearly dependent on pY142-CD3ζ levels. There may be an additional regulatory step in T cell triggering at the step of ZAP70 binding, which should also be tested with other signaling proteins by reconstitution.

In general, protein clustering promotes molecular interactions. However, our observations suggest that the consequence of protein clustering is not necessarily a simple extrapolation from diffusion-limited reactions. It is more complicated in the case of Lck-CD45-TCR networks, because autophosphorylation and modulation of Lck catalytic activities occurs during clustering. Many other protein clusters have been discovered in various cellular systems. Thus, biophysical understanding of non-linear effects of protein clustering remains an important consideration in understanding such signaling mechanisms.

Acknowledgments—We thank L. Huang and X. Li (National Institutes for Materials Science) for the involvement in the early genesis of the work, and R. Vale and E. Hui (University of California, San Francisco) and T. Yamazaki (National Institutes for Materials Science) for helpful discussions. Facilities in the soft material line (National Institutes for Materials Science) were used for experiments.
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