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

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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

Background: The roles of protein clustering in T cell signaling are poorly understood.

Results: Lck clustering recreates conditions in which a phosphatase has positive and negative roles in signal initiation.

Conclusion: Autoinhibition due to Lck clustering is relieved by an optimal concentration of CD45.

Significance: 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 dual positive and negative role in signal initiation.

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) with a 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 Tyr398, 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

The abbreviations used are: TCR, T cell receptor; ITAM, immunoreceptor tyrosine-based activation motif; SFK, Src family tyrosine kinase; SH2, Src homolog domain 2; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphatidylserine; biotin-cap-PE, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine-N-(cap biotinyl); DGS-NTA(Ni), 1,2-dioleoyl-sn-glycero-3-(N-(5-aminol-1-carboxypentyl)iminodiacetic acid)succinyl]; DII, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate; TRITC, tetramethylrhodamine thio-carbamat-
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Lck proteins were expressed in S9 cells using a bac-to-bac system (Invitrogen). Full-length human Lck and Y505F mutant genes conjugated to His<sub>10</sub> 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 His<sub>10</sub> or α-synuclein at the N terminus, cytoplasmic domain of human CD45 (amino acids 584–1281) fused to His<sub>10</sub> at the N terminus and fluorescent proteins (TagBFP 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) and 95% DOPC for the His<sub>10</sub>-proteins system, 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 MgCl<sub>2</sub> was then added to pre-cluster lipids in supported bilayers (for both syn-protein clusters and His<sub>10</sub>-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 Mg<sup>2+</sup> prior to protein conjugation, we prevented or minimized the formation of new protein clusters at later time points when Mg<sup>2+</sup> 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 MgCl<sub>2</sub> 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.
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 (%00 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, fluorescence intensities were calibrated with fluorescence 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 k2. 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.

Protein clusters in the digital images were detected semiautomatically 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 signal. 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 = (0.22w2)/t0.5 (t0.5 = the recovery half-time obtained from the exponential fit of the curve, 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 (Deff) 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.
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 NaHPO4, 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 Tyr325 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 level 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 kcat 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. phosphatidylglycerol or phosphatidylserine), and that clustering was enhanced by divalent cations (e.g. Ca2+ or 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 (phosphatidylserine or phosphatidylglycerol) or cations (Ca2+ or Mg2+) were 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-
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 His\textsubscript{10} tags are attached to DGS-NTA(Ni) in supported lipid bilayers. Lck phosphorylates CD3\zeta as well as Lck (autophosphorylation), and CD45 dephosphorylates both CD3\zeta and Lck. TIRF imaging of the binding of fluorescent probes (labeled antibody and ZAP70) to phospho-CD3\zeta facilitates dynamic monitoring of CD3\zeta phosphorylation.

B, schematic drawing of Lck activity mediated by Tyr505 phosphorylation/dephosphorylation and the formation/release of autoinhibitory structure.

C, FRAP analysis of His\textsubscript{10} proteins (Alexa 647-His\textsubscript{10}-CD3\zeta, Alexa 546-His\textsubscript{10}-Lck, and His\textsubscript{10}-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 m\textsuperscript{2}/s (His\textsubscript{10}-CD3\zeta), 0.64 m\textsuperscript{2}/s (His\textsubscript{10}-Lck), and 0.54 m\textsuperscript{2}/s (His\textsubscript{10}-CD45).

D, monitoring Lck-catalyzed phosphorylation of CD3\zeta. Time-lapse TIRF images of Alexa 488-labeled anti-pY142-CD3\zeta antibody binding to phosphorylated CD3\zeta on supported bilayers (left) and the normalized fluorescence intensities of those images (solid line, right). Protein densities of CD3\zeta and Lck: 300 μm\textsuperscript{2}/2.

E and F, kinetic analysis of CD3\zeta phosphorylation on supported bilayers at an average Lck density = 1.44 μm\textsuperscript{-2} (E) or lower (undetectable by fluorescence) (F). Data were fitted with an allosteric sigmoidal curve and two-dimensional kinetic parameters (k\textsubscript{cat} = 0.21 s\textsuperscript{-1}, K\textsubscript{m} = 203.7 μm\textsuperscript{-2} (E) or 86.5 μm\textsuperscript{-2} (F) and Hill constant, n\textsubscript{H} = 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 k\textsubscript{cat} and the catalytic activity (k\textsubscript{cat}/K\textsubscript{m}) for the data in F. Error bars represent S.E. pY142-CD3\zeta, phosphorylated Tyr\textsuperscript{142} in CD3\zeta.
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 the clusters. More detailed information on protein cluster organization may be provided by higher resolution images. However, these results suggest that syn-Lck has greater affinity for lipids and other α-synuclein tags than syn-CD3ζ. Accordingly, syn-Lck mobility was very low over a wide range of Lck densities as indicated by FRAP (data not shown), and syn-Lck and syn-CD3ζ did not appear to separate from each other (Fig. 2A).

We sought to create another type of protein cluster containing diffusive Lck. We found that 5% DGS-NTA(Ni) in bilayers without PS also clustered in the presence of Mg²⁺ (5 mM MgCl₂) in low salt buffer (30 mM HEPES-NaOH, pH 7.4), and that His10-proteins accumulate in NTA(Ni) lipid clusters (Fig. 2D). FRAP analysis indicated that His10-proteins facilitate a second type of protein cluster, where all proteins are condensed but also diffusive (Fig. 2D). His10-proteins in such clusters had substantial mobile fractions (15–33% 5 min after photobleach) and D_eff ranged from 0.14 to 0.55 μm²/s. We speculate that the NTA(Ni) lipids were phase-separated but were diffusive, and His10-proteins were anchored to these diffusive lipids but did not interact strongly with other proteins, making the proteins diffusive. Note that the affinity between NTA(Ni) and Mg²⁺ may be weaker than that between PS and Mg²⁺, and that competition of the two interactions leads to the partial segregation of DGS-NTA(Ni) from PS-Mg²⁺ clusters in bilayers containing both lipids (Fig. 2A).

Clustered syn-Lck and His10-Lck had distinct mobility and may model two different populations of Lck in T cell membranes. More generally, in these two distinct conditions, we were able to analyze the effects of molecule density and mobility, two key parameters that modulate protein clustering.

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| Membrane system   | n_{cat} | \( k_{cat} \) | \( K_m \) | \( \frac{k_{cat}}{K_m} \) |
|-------------------|---------|---------------|-----------|-------------------------|
| Supported bilayer | 4.2     | 0.21 \( \mu m^2 \) | 204       | 0.013 \( \times 10^{-5} \) μm² |
| Liposome (24)     | 2.3     | 3.41          | 245       | 1.39                    |
| In solution (25)  | 0.0002–0.0098 | 2.3–21.7 \( (\times 10^{-5} \) m² | 1.8–121.8 \( (m^{-1} s^{-1}) \) |

**TABLE 1**

Two-dimensional kinetic parameters of Lck on membranes

The result in Fig. 1E was compared with representative data for the two-dimensional (on liposome (24)) and three-dimensional (in solution phase (25)) kinetic parameters. Two-dimensional kinetic parameters of Lck on membranes

In Vitro Reconstitution of T Cell Signaling Protein Clusters

The net CD3ζ phosphorylation activity in the CD3ζ-Lck-CD45 network was measured in two distinct protein clusters, syn-protein clusters and His10-protein clusters, and in homogeneous membranes. We measured near steady-state levels of CD3ζ phosphorylation in a range of protein densities when the reactions proceeded substantially (8 min, Fig. 2C). Fluorescence intensities of Alexa 647-CD3ζ, Alexa 546-Lck, CD45-TagBP, and Alexa 488-anti-pY142-CD3ζ antibody were obtained through four-color imaging, and calibrated to estimate the densities of CD3ζ, Lck, and CD45 (Fig. 2A). Ratios of fluorescence intensity of anti-pY142-CD3ζ to CD3ζ densities (p-CD3ζ/CD3ζ) were taken to reflect CD3ζ phosphorylation levels. Protein densities were distributed among clusters even in the same bilayer, and data from multiple bilayers were collected for the analysis.

We observed CD45 inhibition of CD3ζ phosphorylation in the context of the CD3ζ-Lck-CD45 network in all His10-protein systems, either in homogeneous membranes or in clusters (Fig. 3, A and B). Near physiological Lck densities (~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
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dehosphorylation of the inhibitory tyrosine 505 (Fig. 1B), we examined mutant syn-Lck Y505F that lacked Tyr<sup>505</sup> 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 Tyr<sup>505</sup> 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. Tyr505 phosphorylation in syn-Lck clusters decreased monotonically with increasing CD45 density, but was preserved at high CD45 densities, indicating rapid autophosphorylation of Tyr505 in syn-Lck clusters that competes with dephosphorylation by CD45 (Fig. 4A). 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 Tyr505 enhances Lck catalytic activity (1), and we observed robust Tyr505 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 higher 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 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 intramolecular 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 $K_m$ for ATP than Tyr394/394.
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 CD3/H9256, resulting in a net positive regulation of CD3/H9256 phosphorylation. Conversely, diffuse Lck autophosphorylates exclusively at Tyr^{394}, a positive regulatory site, and thus CD45 acts only negatively on both Lck and CD3/H9256. Therefore, the phosphorylation level of

**FIGURE 3.** Steady-state phosphorylation of CD3ζ in the reaction network with Lck and CD45 in differentially reconstituted clusters and homogeneous membranes. The CD3ζ phosphorylation level was plotted as the ratio to the total CD3ζ density (pY142-CD3ζ/CD3ζ) at different Lck and CD45 densities. A and B, CD3ζ phosphorylation levels in the network of His_{10}-proteins in homogeneous membranes (A) and diffusive His_{10}-proteins in clusters (B) were plotted following normalization to the phosphorylation levels of CD45 = 0 μm^{-2}. Lipid compositions in supported bilayers: 5% DGS-NTA(Ni) and 95% DOPC. C, CD3ζ phosphorylation levels in A and B at different Lck densities in the absence of CD45 (CD45 = 0 μm^{-2}) compared and normalized in a single scale. D, CD3ζ phosphorylation levels in the syn-protein clusters were plotted after normalization to the phosphorylation levels of CD45 = 0 μm^{-2}. Lipid composition of supported bilayers: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC. E, normalized CD3ζ phosphorylation levels in syn-protein clusters containing syn-Lck-Y505F mutant proteins. Bilayer compositions are the same as in D. F, CD3ζ phosphorylation levels in clusters containing either syn-Lck (wild-type) or syn-Lck-Y505F at different Lck densities, in the absence of CD45 (CD45 = 0 μm^{-2}). D and E, were compared and normalized in a single scale. G, phosphorylated CD3ζ levels in syn-protein clusters that were further normalized with Lck densities plotted at different cluster sizes. H, detection of CD3ζ phosphorylation in syn-protein clusters by the AcGFP-tagged tandem SH2 domain of ZAP70. Reconstituted protein clusters contained α-synuclein (syn-) tagged CD3ζ (Alexa 647-labeled) and Alexa 546-labeled syn-Lck, and partially excluded His_{10}-CD45-TagBFP. Lipid compositions in supported bilayers were: 5% DGS-NTA(Ni), 25% DOPS, and 70% DOPC. I, AcGFP-ZAP70 levels in syn-clusters plotted on different Lck densities in the absence of CD45. The fluorescent signal was compared with the background signal of outside clusters to minimize the effect of the relatively higher background signal of ZAP70, caused by nonspecific binding to NTA(Ni) in the bilayer.
CD3e 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.

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FIGURE 5. Model of TCR-Lck-CD45 network regulation by molecular clustering. A. Lck autophosphorylates Tyr394 with a lower ATP Km than for Tyr505 autophosphorylation. Tyr394 autophosphorylation preferably occurs in trans (intermolecularly), whereas Tyr505 autophosphorylation may occur both in trans and cis (intramolecular reaction). In immobilized Lck proteins, an intramolecular interaction is kinetically more favorable than an intermolecular interaction. Thus, in immobilized Lck, Tyr505 autophosphorylation could occur intramolecularly, whereas diffusive Lck exclusively autophosphorylates Tyr394 in trans. B, when TCR clusters are enriched with Lck containing Tyr(P)505, such as immobilized Lck that autophosphorylates Tyr505 as in A, dephosphorylation of Tyr(P)505 by CD45 increases the catalytic activity of Lck (positive effect), whereas CD45 also dephosphorylates TCR (negative effect). At low CD45 densities, the positive effect could overcome the negative effect, resulting in a net positive regulation by CD45 in TCR phosphorylation. Conversely, TCR clusters containing diffusive Lck with Tyr(P)394 as in A were negatively regulated at all CD45 densities. C, CD3ζ phosphoryrosines in the cluster interact with the Lck SH2 domain and recruits Lck. Recruited Lck molecules in the cluster are trapped and immobilized, and autophosphorylate Tyr505 in cis. The Tyr(P)505 interacts intramolecularly with the SH2 domain that competes with the intermolecular phosphorylated CD3ζ-Lck interaction, resulting in the release of Lck from the cluster. Released Lck lacking Tyr(P)505 autophosphorylation activity is dephosphorylated by CD45. At equilibrium conditions, clusters are enriched with pYS505-Lck, but not with all Lck.
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