

Genetically Encoded Förster Resonance Energy Transfer Sensors for the Conformation of the Src Family Kinase Lck¹

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The current model for regulation of the Src family kinase member Lck postulates a strict correlation between structural condensation of the kinase backbone and catalytic activity. The key regulatory tyrosine 505, when phosphorylated, interacts with the Src homology 2 domain on the same molecule, effectively suppressing tyrosine kinase activity. Dephosphorylation of Tyr⁵⁰⁵ upon TCR engagement is supposed to lead to unfolding of the kinase structure and enhanced kinase activity. Studies on the conformation-activity relationship of Lck in living cells have not been possible to date because of the lack of tools providing spatiotemporal resolution of conformational changes. We designed a biochemically active, conformation-sensitive Förster resonance energy transfer biosensor of human Lck using the complete kinase backbone. Live cell imaging in Jurkat cells demonstrated that our biosensor performed according to Src family kinase literature. A Tyr⁵⁰⁵ to Phe mutation opened the structure of the Lck sensor, while changing the autophosphorylation site Tyr³⁹⁴ to Phe condensed the molecule. The tightly packed structure of a high-affinity YEEI tail mutant showed that under steady-state conditions the bulk of Lck molecules exist in a mean conformational configuration. Although T cell activation commenced normally, we could not detect a change in the conformational status of our Lck biosensor during T cell activation. Together with biochemical data we conclude that during T cell activation, Lck is accessible to very subtle regulatory mechanisms without the need for acute changes in Tyr⁵⁰⁵ and Tyr³⁹⁴ phosphorylation and conformational alterations. *The Journal of Immunology*, 2009, 182: 2160–2167.

One of the earliest biochemical events following TCR triggering is tyrosine phosphorylation of ITAMs within the CD3 and TCR ζ signaling chains (1, 2). Phosphorylated ITAMs are key docking sites for downstream mediators of T cell signaling (3). Pioneering work in transformed T cells found the Src family kinase (SFK)⁵ Lck indispensable for this early wave of tyrosine phosphorylation (4), and Lck-deficient mice exhibited severely impaired T cell development with almost no detectable CD4 and CD8 single positive T cells (5). Despite years of ongoing

research on Lck, the immediate events and posttranslational modifications on Lck leading up to ITAM phosphorylation are poorly understood. Both intracellular localization and catalytic activity are thought to regulate the initiation of downstream signaling events of Lck. In line with this, biochemical and microscopic studies suggested that Lck is recruited to the site of TCR clustering during the first couple of minutes following TCR engagement (6, 7). Besides these observations, an increase in catalytic activity, controlled by phosphorylation status and intramolecular interactions, is currently believed to be the main regulatory element for Lck action. In the auto-inhibited state of Lck, the Src homology (SH) 3 and SH2 domains turn inward and pack against the catalytic domain on the side opposite to the catalytic cleft. Although the SH2 and SH3 domains do not sterically block the catalytic domain in the autoinhibited state, the catalytic domain itself is locked in an inactive conformation via distortions of the active site (8). Three critical components regulate the catalytic activity of Lck, referred to as the “switch,” the “clamp,” and the “latch” (9). The latch is the C-terminal tail, which bears the autoinhibitory phosphorylation site Tyr⁵⁰⁵. When phosphorylated, Tyr⁵⁰⁵ interacts with the SH2 domain of Lck, which effectively suppresses tyrosine kinase activity (10–13). Interactions between the SH3 domain and the linker connecting the SH2 domain to the catalytic domain build up the clamp and play an additional essential role in stabilizing the autoinhibited state (14). Suppressive phosphorylation of Lck at Tyr⁵⁰⁵ is accomplished by the tyrosine kinase C-terminal Src kinase (Csk) (15). Dephosphorylation of Tyr⁵⁰⁵, most probably by the tyrosine phosphatase CD45, opens the latch and removes intramolecular inhibition followed by activation of the kinase (16). Besides dephosphorylation of Tyr⁵⁰⁵, full kinase activity additionally requires the phosphorylation of the positive regulatory Tyr³⁹⁴ situated in the activation loop of the catalytic domain (17). Phosphorylation of Tyr³⁹⁴ operates the switch and promotes the active position of the C helix within the small lobe of the catalytic

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⁵ Abbreviations used in this paper: SFK, Src family kinase; *Eapp*, apparent FRET efficiency; CLckY, construct ECFP-Lck-EYFP; CLck-2, donor-only tagged Lck; LckY, acceptor-only tagged Lck; ECFP, fused ECFP and EYFP; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, Förster resonance energy transfer; pTyr, phosphotyrosine; SH, Src homology (domain); TIRF, total internal reflection fluorescence.

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domain. This rearrangement brings the key catalytic residues Glu³¹⁰ and Lys²⁹⁵ into the catalytic cleft, which coordinate the phosphates of the bound ATP (18).

Visualization of the proposed structural changes of Lck should enable us to follow its activation, time and space resolved, directly in the living T cell. Förster resonance energy transfer (FRET) is a suitable method for detecting structural changes (19). FRET describes the nonradiative energy transfer from the electronically excited state of a donor dye molecule to an acceptor dye molecule. Its efficiency sharply increases with decreasing donor-acceptor distance below a critical distance (referred to as the Förster radius), making FRET an ideal readout parameter of a conformation-sensitive molecular biosensor (20, 21). We report in this work the design of a genetically encoded unimolecular FRET sensor for Lck consisting of the full Lck backbone with enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) insertions. The rationale behind this approach was to preserve the biochemical activity of the chimerical molecule, enabling us to directly monitor kinase structure during activation. Similar approaches were successfully realized for the kinases MK2 and Akt (22, 23).

Our Lck sensor constructs allowed us for the first time to resolve different conformations of the kinase structure in living T cells. Despite the proven conformation sensitivity of our biosensor toward changes in Tyr⁵⁰⁵ and Tyr³⁹⁴, we did not detect changes in FRET efficiency during TCR engagement. Supported by biochemical data, we exclude a role for acute Lck tail dephosphorylation and structural changes during proximal TCR signaling. It rather seems that the bulk of Lck molecules exist in a mean conformational configuration, as shown by introducing a high-affinity tail sequence to our biosensor.

Materials and Methods

Antibodies

CD3 mAbs (MEM-92 and MEM-57) were a gift from Dr. V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Mouse anti-phosphotyrosine (pTyr) mAb 4G10 was purchased from Upstate Biotechnology. Rabbit anti-phospho-Y505-Lck, anti-phospho-Y416-Src, and anti-Lck polyclonal Abs were purchased from Cell Signaling.

Cell lines and constructs

The wild-type Jurkat T cell line E6.1 was purchased from American Type Culture Collection. The Lck-deficient Jurkat T cell line J.CaM1.6 was obtained from the European Collection of Cell Cultures (Salisbury, U.K.). Lck biosensor constructs were prepared in the retroviral expression vector pBMN-Z (provided by G. Nolan, Stanford University School of Medicine, Stanford, CA) using standard molecular biology methods. Production of viral supernatants was performed using Phoenix-gp cells (provided by G. Nolan). Jurkat T cells were transduced with 48-h Phoenix-gp cell supernatant and fluorescence protein positive cells were sorted for high expression using a FACSaria (BD Biosciences) cell sorter. ECFP consists of ECFP and EYFP fused by a 27-aa-long spacer. It was a gift from Dr. J. Schmid (Institute of Vascular Biology, Centre of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Vienna).

Analysis of protein tyrosine phosphorylation

Before stimulation, Jurkat T cells were incubated in RPMI 1640 medium supplemented with 1% FCS for 4 h at 37°C. Cells (6×10^5) were stimulated for different time points at 37°C using the CD3 mAb MEM-92 (10 μ g/ml). The reactions were stopped by the addition of ice-cold washing buffer (20 mM Tris-HCl (pH = 7.5), 150 mM NaCl, and 5 mM EDTA). After centrifugation (for 2 min at $850 \times g$ and 4°C), cells were immediately lysed for 30 min in ice-cold lysis buffer (20 mM Tris-HCl (pH = 7.5), 150 mM NaCl, and 2 mM EDTA) containing 1% Nonidet P-40 (Pierce), 1 mM sodium orthovanadate, 20 mM NaF, and protease inhibitors (5 mM aprotinin, 5 mM leupeptin, 1 mM PMSF, and 1 M pepstatin; all from Sigma-Aldrich). After centrifugation (for 5 min at $14,000 \times g$ and 4°C),

lysates were analyzed by reducing SDS-PAGE conditions (12% gel) and immunoblotting using an HRP-labeled anti-pTyr mAb, 4G10.

Stimulatory surface for life cell imaging

A thin perfusion chamber was mounted onto an aldehyde modified no. 1 glass slide (150 μ m) homogeneously coated with a layer of protein A (50 μ g/ml; catalog no. P 3838, Sigma-Aldrich) and the CD3 mAb MEM-57 (5 μ g/ml). All experiments were performed at 37°C and adjusted by an objective heating system (PeCon).

Total internal reflection fluorescence (TIRF) microscopy/FRET imaging

The beam of a 35-milliwatt, 405-nm laser and the 514-nm line of an Innova 70C multiline laser (both from Coherent) were coupled into an inverted epifluorescence microscope (Axiovert 200; Zeiss) via a custom-made triple band dichroic (AHF-Analysentechnik). Fluorescence was excited and collected via a $\times 100$ α -Plan Neofluar objective (Zeiss) that was mounted on a focus hold system (patent A 1155/2003). The microscope was equipped with a modified total internal reflection condenser (TILL-Photonics) that allowed for switching between conventional Köhler and total internal reflection illumination. Assuming a refractive index for Jurkat cytosol of $n \sim 1.38$, a penetration depth of the evanescent field of 72 nm can be estimated (24).

For FRET imaging, a set of three images was recorded for both donor dequenching and sensitized emission imaging. First, the donor was excited at 405 nm and both donor emission (I_{DD} ; bandpass 480/20) and acceptor emission (I_{DA} ; bandpass 550/40) were detected after splitting by a dichroic beamsplitter Q505LP (all from AHF-Analysentechnik) using two charge-coupled device cameras (CoolSNAP_{HQ}; Roper Scientific). Second, the acceptor was excited at 514 nm and the acceptor emission (I_{AA} ; bandpass 550/40) was detected. Images were recorded as large scans for statistical analysis of many cells using time delay and integration readout (25). For large area scans an illumination time per line of 1 ms was chosen in the time delay and integration readout mode, resulting in a total illumination time of 260 ms. Binning (4×4) resulted in a pixel size of 258 nm in all images.

FRET analysis

Analysis of FRET-efficiencies (E) from fluorescence images was conducted in two different modes: 1) Donor dequenching by acceptor photobleaching; and 2) sensitized emission FRET imaging as described recently (26).

In brief, for donor dequenching, images of donor emission at donor excitation (I_{DD}) were acquired before (I_{DD}^{pre}) and after (I_{DD}^{post}) acceptor photobleaching (using 43-fold higher excitation intensity for a duration of 60 s). E was calculated according to Equation 1:

$$E = \frac{I_{DD}^{post} - I_{DD}^{pre}}{I_{DD}^{post}} \quad (1)$$

For three-cube sensitized emission FRET imaging we used an E calibration method introduced by Zal and Gascoigne (27) that allows for monitoring E over time. The corrected sensitized emission signal F_c was obtained by Equation 2:

$$F_c = I_{DA} - aI_{AA} - dI_{DD} \quad (2)$$

In this formula, the crosstalk of the donor signal into the acceptor channel (dI_{DD}) and the direct excitation of the acceptor at the donor excitation wavelength (aI_{AA}) are taken into account. On cell lines expressing ECFP or EYFP only, we determined $d = 0.33$ and $a = 0$ (no direct excitation of EYFP at 405 nm).

Determination of a setup calibration factor, shown in Equation 3,

$$G = \frac{F_c}{I_{DD}^{post} - I_{DD}} \quad (3)$$

enabled us, as shown in Equation 4,

$$E = \frac{F_c}{F_c + GI_{DD}} \quad (4)$$

to directly relate F_c to E . For reliable determination of G we conducted acceptor photobleaching on the ECFP construct exhibiting a FRET efficiency of $25 \pm 6\%$ ($n = 95$ cells) that yielded $G = 2$.

Video microscope measurements of intracellular calcium fluxes

Cells were loaded with 10 μ M Indo-1/AM (Molecular Probes) in culture medium at room temperature for 15 min, washed with HBSS (Invitrogen)

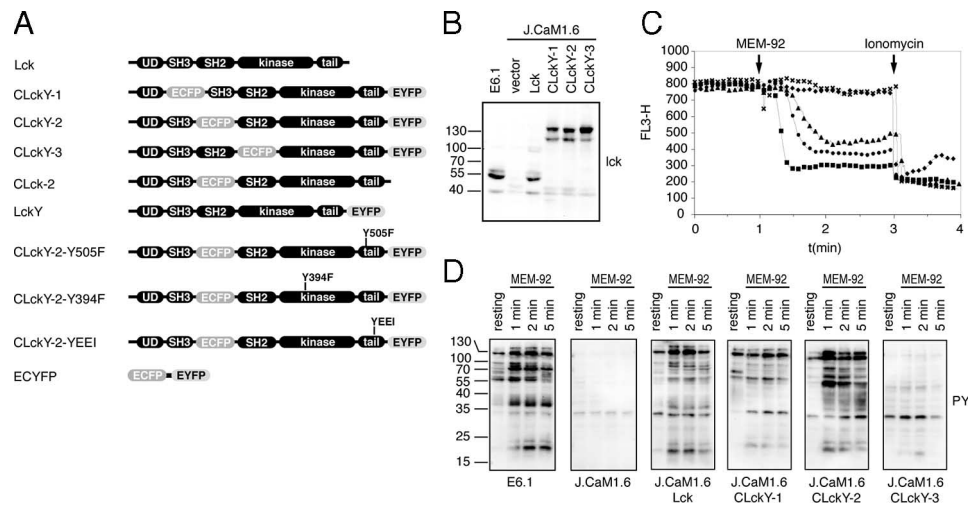


FIGURE 1. Design and characterization of the Lck biosensors. *A*, Schematic representation of the different Lck and control constructs used in this study. UD, Unique domain. *B*, Expression levels of the FRET constructs CLckY-1, CLckY-2, and CLckY-3 in J.CaM1.6 were compared with the wild-type Jurkat clone E6.1 and with J.CaM1.6 transduced with wild-type Lck (construct “Lck”) or the empty vector by Western blotting. *C*, Calcium fluxes as measured by flow cytometry. J.CaM1.6, untransduced or transduced with the different Lck constructs, were loaded with the calcium-sensitive dye fura red. Calcium transients in response to the CD3 mAb MEM-92 and the calcium ionophore ionomycin were measured by flow cytometry. Wild-type Jurkat E6.1 (squares), Lck-deficient Jurkat J.CaM1.6 (diamonds), J.CaM1.6 transduced with CLckY-1 (triangles), CLckY-2 (circles), and CLckY-3 (crosses) are shown. *D*, Western blot analysis of tyrosine phosphorylation. Jurkat E6.1 and J.CaM1.6 cells, untransduced or transduced with the different Lck constructs, were stimulated with CD3 mAb MEM-92 for the indicated times. Cells were pelleted and solubilized and the cell lysates were resolved by 12% SDS-PAGE and immunoblotted for tyrosine phosphorylation (PY).

and flushed into hybridization chambers (Invitrogen) on the stage of a Zeiss Axiovert microscope. Cells were illuminated using a mercury lamp (Zeiss) at 333 nm, and fluorescence emissions at 405 and 485 nm were detected simultaneously by two charge-coupled device cameras (all bandpass filters and dichroic mirrors from Chroma Technology). Videos were acquired and the emission ratio of 485/405 nm was determined.

FACS analysis of intracellular calcium fluxes

Jurkat T cells were loaded with Fura Red (2 μ M; Molecular Probes) for 30 min at room temperature. After two rounds of washing, cells were rested on ice for at least 15 min. Before measurement, cells were equilibrated at 37°C for 5 min. Changes in intracellular calcium were monitored using a FACSCalibur (BD Biosciences) analyzer. First, cells were acquired for 1 min without stimulation to monitor baseline activity, then CD3 mAb MEM-92 was added (10 μ g/ml) and cells were acquired for another 3 min. As a control, the calcium ionophore ionomycin (1 μ M) was added and cells were acquired for one more minute.

Results

Biochemical activity of chimerical Lck molecules

To enable a direct assessment of conformation, we based the design of our Lck sensors on the complete kinase molecule. All of our FRET sensor constructs carry the acceptor fluorophore EYFP at the C terminus, following the regulatory Tyr⁵⁰⁵. The position of the donor fluorophore ECFP was varied to define the best position for maximum FRET changes and minimum interference with structure and function. Construct ECFP-Lck-EYFP (CLckY)-1 (CLckY-1) carries the ECFP insertion closest to the N terminus, between the unique and SH3 domains. Constructs CLckY-2 and CLckY-3 contain the ECFP at the linker regions between the SH3 and SH2 domains and the SH2 and catalytic domains, respectively. The N terminus is critical for membrane targeting of Lck via myristoylation (28) and palmitoylation (29) and thus was left untouched in all constructs (Fig. 1*A*).

To assess the biochemical activity of the tagged Lck molecules, we performed reconstitution experiments in the Lck-deficient Jurkat cell line J.CaM1.6. This T cell line shows very low tyrosine phosphorylation and Ca²⁺ flux in response to CD3 mAb stimulation (4). The expression levels of the tagged Lck molecules in

J.CaM1.6 cells were comparable to the endogenous levels in wild-type Jurkat E6.1 (Fig. 1*B*). Constructs CLckY-1 and CLckY-2, but not CLckY-3, reconstituted Ca²⁺ flux and tyrosine phosphorylation in response to CD3 mAb stimulation (Fig. 1, *C* and *D*). CLckY-2 was closest to Lck wild-type in reconstituting J.CaM1.6 and was chosen for the additional experiments.

Live cell imaging of the Lck biosensor

For live cell imaging, we applied Jurkat T cells to a CD3 mAb-coated glass surface in a temperature-controlled perfusion chamber (referred to as “stimulatory surface”). Under this regimen, wild-type Jurkat E6.1 showed all the hallmarks of T cell activation such as calcium transients, tyrosine phosphorylation, and cell spreading (30) (supplemental Fig. 1, *A* and *B* online).⁶ J.CaM1.6 cells showed a very small response to the stimulatory surface, which is in accord with the defect of these cells in forming conjugates with APCs (31). Consistent with the biochemical results, construct CLckY-2 reconstituted responsiveness of J.CaM1.6 (supplemental Fig. 1*C* online).

Next, we analyzed the construct CLckY-2 for the occurrence of FRET by donor dequenching after acceptor photobleaching. We confined the illumination to the contact plane of the T cell with the stimulatory surface by TIRF microscopy (24) under dual color illumination. Five minutes after contact formation with the stimulatory surface, complete acceptor photobleaching of CLckY-2-expressing cells was performed. We obtained a robust increase in donor intensity corresponding to a FRET efficiency *E* value of $19 \pm 5\%$ ($n = 21$ cells; Fig. 2*A*, left panel). For comparison, we measured the FRET positive control construct ECFYP, which consists of ECFP and EYFP fused via a short peptide linker (Fig. 1*A*) and found an *E* value of $25 \pm 6\%$ ($n = 95$ cells; Fig. 2*A*, right panel). Thus, our Lck biosensor CLckY-2 exhibits high FRET efficiency when imaged under TIRF illumination in the membrane of T cells.

⁶ The online version of this article contains supplemental material.

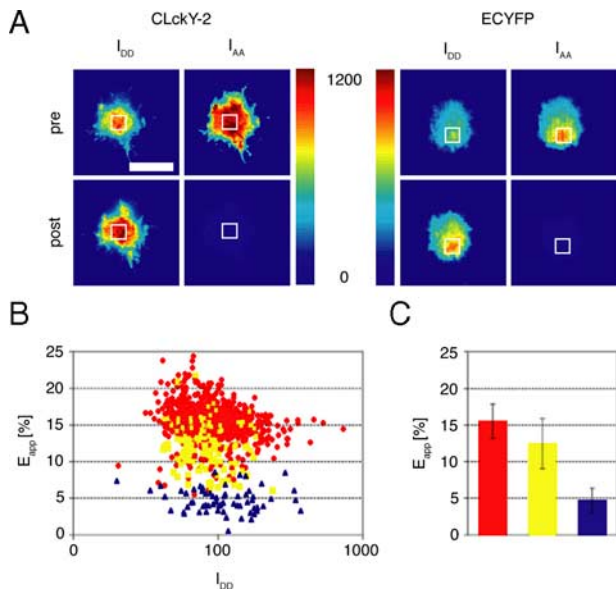


FIGURE 2. FRET-imaging of the Lck biosensor. **A**, Membrane-proximal FRET efficiency of the Lck biosensor construct CLckY-2 and the FRET control construct ECFP as determined by donor dequenching after acceptor photobleaching under TIRF illumination. Constructs CLckY-2 and ECFP were expressed in the Lck-deficient T cell line J.CaM1.6 and in Jurkat E6.1, respectively. Pseudo-colored images of donor and acceptor emission before (pre) and after (post) acceptor photobleaching of the entire cell are shown. Mean FRET efficiency values were calculated using Equation 1 (see *Materials and Methods*) from mean I_{DD} intensities of regions of interest (white rectangles); $E = 19 \pm 5\%$ ($n = 21$) for CLckY-2 and $E = 25 \pm 6\%$ ($n = 95$) for ECFP. The color bar illustrates the translation of fluorescence intensity into a color scale with a maximum of 1200 counts. Scale bar 10 μm . **B**, Discriminating between intramolecular and intermolecular FRET of the biosensor CLckY-2. FRET efficiencies, E_{app} , of individual cells expressing biosensor constructs were determined over a range of expression levels (I_{DD}). In contrast to direct determination of E via donor dequenching as used in Fig. 2A, we used three-cube sensitized emission imaging. After subtraction of background, single-cell averaged intensities were used to calculate E_{app} according to Equation 4 (see *Material and Methods*). All measurements were performed on stimulatory CD3 mAb-coated surfaces 5 min after contact formation of the cells with the glass surface. CLckY-2 was either expressed in J.CaM1.6 (red diamonds) or Jurkat E6.1 (yellow squares). The single tagged molecules CLck-2 (ECFP only) and LckY (EYFP only) were coexpressed in J.CaM1.6, and only cells with a molar ratio of donor and acceptor between 1:2 and 2:1 were analyzed (blue triangles). Each dot represents an individual cell; $n = 903$ for CLckY-2 in J.CaM1.6; $n = 135$ for CLckY-2 in E6.1; and $n = 65$ for CLck-2/LckY in J.CaM1.6. **C**, Comparison of the mean FRET efficiencies, E_{app} , of the three different cohorts of cells measured in Fig. 2B (15.5 ± 2.3 , 12.5 ± 3.4 , and $4.7 \pm 1.6\%$, respectively).

The Lck biosensor is conformation sensitive

The high FRET values of CLckY-2 may have two contributions: conformation-dependent intramolecular FRET and intermolecular FRET caused by the close proximity of fluorescent protein-tagged molecules in the membrane. To obtain statistically relevant results, we analyzed scans of large surface areas containing many cells. Although donor dequenching after acceptor photobleaching is a robust method, it is quite tedious and time consuming, making it unsuitable for large-scale analysis of cells. To overcome this problem, we implemented three-cube sensitized emission imaging as introduced by Zal and Gascoigne (27), which allowed for direct calculation of FRET efficiencies (apparent FRET efficiencies (E_{app})) on a large cohort of cells without applying acceptor photobleaching (26).

To discriminate between intramolecular and intermolecular FRET contributions, we first tested the dependence of the FRET efficiency on Lck expression levels; we reasoned that accidental proximity would increase with the average density of the tagged Lck molecules in the membrane, thereby leading to an increase in FRET efficiency. J.CaM1.6 cells with varying expression levels of the biosensor CLckY-2 were analyzed. The FRET efficiency was found to be largely independent of the expression level (Fig. 2B, red diamonds), yielding an average E_{app} of $15.5 \pm 2.3\%$ ($n = 903$ cells). Second, we measured the contribution of intermolecular FRET due to close proximity of nonrandomly distributed, e.g., clustered, CLckY-2. For this, we expressed CLckY-2 in Jurkat E6.1 to compete with unlabeled wild-type Lck molecules. We obtained E_{app} values of $12.5 \pm 3.4\%$ ($n = 135$ cells) over the whole expression range (Fig. 2B, yellow squares) when measuring on a wild-type background. Third, we coexpressed a donor-only tagged Lck (CLck-2) and an acceptor-only tagged Lck (LckY) in J.CaM1.6. Donor and acceptor in the single-tagged constructs are in the same positions as in the double-tagged CLckY-2, providing a similar environment for intermolecular FRET. For analysis, we selected cells with a molar ratio of CLck-2 and LckY between 1:2 and 2:1, which is close to the exact 1:1 ratio of the dyes in the CLckY-2 construct. We found a low FRET efficiency of $E_{app} = 4.7 \pm 1.6\%$ ($n = 65$ cells; Fig. 2B, blue triangles). Together, these three independent experiments indicate that intramolecular energy transfer represents the dominant contribution to the observed high FRET values of construct CLckY-2.

Monitoring FRET efficiency of the Lck biosensor during T cell activation

All measurements until now were done in a standardized way, 5 min after contact formation of the cells with the stimulatory surface. Taking into account the dynamics of Tyr phosphorylation, which peaks between 1 and 2 min after T cell stimulation (Fig. 1D), we determined the time course of FRET efficiency during T cell activation. Individual cells were imaged as they approached the stimulatory surface and spread out. During a time period of 2 min we detected a clear increase in donor intensity upon contact formation with the stimulatory surface, however, we did not see a detectable change in FRET efficiency, which remained constantly high throughout the cell spreading process (Fig. 3, A and B). One could argue that TCR clustering on the stimulatory surface increased the proximity of fluorescent protein-tagged Lck molecules and therefore FRET efficiency in the membrane. Such a phenomenon could potentially mask the opening of the Lck biosensor molecules. To see whether clustering-induced changes in intermolecular FRET efficiency occurred, we applied J.CaM1.6 cells coexpressing donor-only tagged Lck, CLck-2, and acceptor-only tagged Lck, LckY, on the stimulatory surface. We did not observe any significant changes in FRET efficiency over time between CLck-2 and LckY molecules, displaying an E_{app} of $4.7 \pm 2.1\%$ ($n = 58$ cells) and $5.1 \pm 2.8\%$ ($n = 61$ cells) at 2 and 10 min, respectively. Based on these data, we propose that the conformation of membrane-associated Lck does not change during TCR signaling (Fig. 3B). This conclusion is further substantiated by measurements of cells under steady-state resting conditions on BSA-blocked glass surfaces. As judged by calcium measurements, the blocked surfaces are nonactivatory (supplemental Fig. 1, A and B, online). Importantly we did not find a difference in FRET efficiency between cells on CD3- or BSA-coated surfaces (E_{app} of $15.6 \pm 2\%$ with $n = 72$ cells or $16.5 \pm 3.3\%$ with $n = 214$ cells; Fig. 3C).

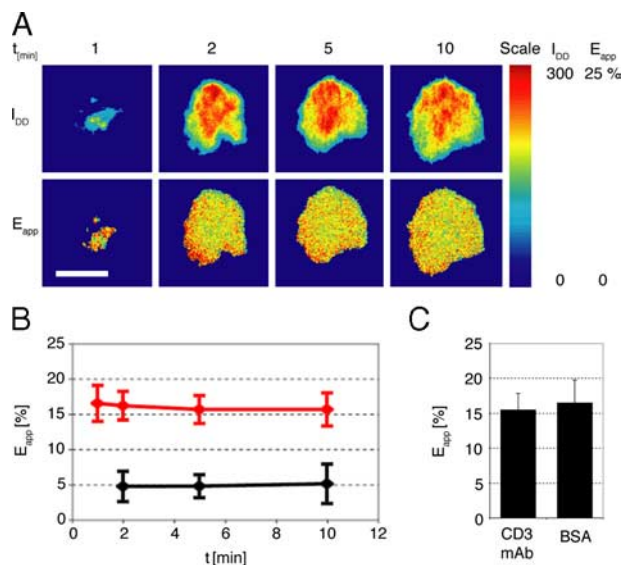


FIGURE 3. FRET efficiency of the Lck biosensor during T cell stimulation. J.CaM1.6 cells expressing the construct CLckY-2 were allowed to settle on a stimulatory surface and imaged under TIRF illumination. *A*, I_{DD} (upper panel) as well as E_{app} (lower panel) were determined at the indicated time points. E_{app} of the typical single cell shown remained constant at $15.5 \pm 0.7\%$. The color bar illustrates the translation of fluorescence intensity and FRET efficiency into a color scale with a maximum of 300 counts and 25%, respectively. Scale bar, 5 μm . *B*, Time course from sequential scans of a large area of J.CaM1.6 cells on a stimulatory surface either expressing CLckY-2 (red line) or coexpressing the single tagged molecules CLckY-2 and LckY (black line). Data are mean $\pm$ SD of at least 30 cells. *C*, FRET efficiencies of J.CaM1.6 expressing CLckY-2 on either stimulatory CD3 mAb or nonstimulatory, BSA-blocked surfaces 5 min after contact formation; $15.6 \pm 2.0\%$, $n = 72$ for CLckY-2 on CD3; and $16.5 \pm 3.3\%$, $n = 214$ for CLckY-2 on BSA.

The data obtained so far is contrary to the currently established model of Lck regulation that postulates acute tail dephosphorylation accompanied by structural unfolding as prerequisites of kinase activation. Therefore, we next wanted to verify that dephosphorylation of Tyr⁵⁰⁵ in the chimerical Lck construct changes the distance between donor and acceptor in a detectable way.

Visualizing the open, Tyr⁵⁰⁵-dephosphorylated form of Lck

To test whether dephosphorylation of Tyr⁵⁰⁵ alters the FRET efficiency of CLckY-2, we introduced a point mutation that changes the C-terminal Tyr⁵⁰⁵ to Phe (construct CLckY-2-Y505F) and thereby disables the regulatory Y505/SH2 latch. This mutation is known to lead to a constitutively active kinase phenotype enhancing T cell responsiveness to Ag (32) and inducing Ag-independent IL-2 production in Th cell hybridomas (33). Indeed, CLckY-2-Y505F was hyperactive as determined by anti-phosphotyrosine blotting (data not shown). E_{app} of the mutant construct was $8.7 \pm 2.4\%$ ($n = 34$ cells) and therefore significantly lower than that of the nonmutated version with $14.9 \pm 2.0\%$ ($n = 90$ cells; Fig. 4A). These data demonstrate that the low FRET values of the Y505F mutant derive from an open and catalytically more active form of the kinase. This indicates that the nonmutated parental construct CLckY-2 is sensitive to quantitative dephosphorylation of Tyr⁵⁰⁵. To address the question of why T cell stimulation did not alter the FRET efficiency of our biosensor in a detectable way, we assessed the phosphorylation of Tyr⁵⁰⁵ during TCR engagement using a pTyr⁵⁰⁵-specific Ab. In agreement with the FRET data, we could not detect significant changes in the phosphorylation content of Tyr⁵⁰⁵ on wild-type Lck or CLckY-2 during early mAb-mediated

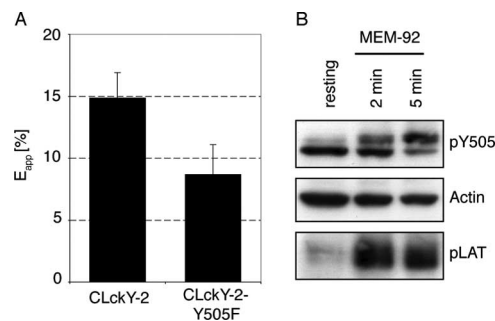


FIGURE 4. Sensitivity of the Lck biosensor to the Y505/SH2-domain regulatory latch. *A*, FRET efficiencies of J.CaM1.6 expressing CLckY-2 or the Tyr⁵⁰⁵ to Phe mutant construct CLckY-2-Y505F. The bar graph depicts mean E_{app} values of cell cohorts analyzed under TIRF illumination 5 min after contact formation with the stimulatory surface; $14.9 \pm 2.0\%$, $n = 90$ for CLckY-2; and $8.7 \pm 2.4\%$, $n = 34$ for CLckY-2-Y505F. *B*, Western blot analysis of TCR signaling. Jurkat E6.1 cells were stimulated with CD3 mAb MEM-92 for the indicated times. Cells were pelleted and solubilized and the lysates were resolved by 12% SDS-PAGE and immunoblotted using anti-phospho-Y505-Lck (pY505; top), anti-actin as loading control (middle), and anti-phospho-LAT (pLAT; bottom).

T cell stimulation (Fig. 4B and data not shown). The appearance of higher m.w. forms of Lck in a pTyr⁵⁰⁵ blot is most likely caused by phosphorylation of Ser⁵⁹ (34).

Visualizing the crosstalk between regulatory and catalytic domains

Lck kinase activity is crucially dependent upon autophosphorylation of Tyr³⁹⁴ on the activation loop within the catalytic domain. Phosphorylation of the corresponding Tyr⁴¹⁶ in the prototypic SFK Src was shown to destabilize the interaction of the SH3 domain with the SH2-CD linker (35). Therefore, we introduced a Tyr³⁹⁴ to Phe mutation in CLckY-2, which is known to strongly decrease kinase activity of wild-type Lck (36). The mutated CLckY-2-Y394F failed to reconstitute the signaling deficiency of J.CaM1.6 cells (Fig. 5A) and showed a very high FRET value of $22.0 \pm 1.5\%$ ($n = 83$ cells; Fig. 5B) and, thus, a more compact conformation compared with the parental CLckY-2 with $15.6 \pm 2.3\%$ ($n = 291$ cells). Therefore, our biosensor is able to visualize the influence of autophosphorylation at Tyr³⁹⁴ on the overall Lck protein structure towards an open catalytically competent conformation despite intact latch and clamp interactions. Together with the data obtained with the CLckY-2-Y505F mutant, our Lck FRET sensor CLckY-2 senses modifications of the two main regulatory Tyr residues, 394 and 505. Because it did not alter its FRET values upon T cell activation, we conclude that acute modifications of the autophosphorylation or regulatory tail tyrosines do not play a dominant role in T cell signaling.

High-affinity tail sequences condense the Lck structure

We further looked into the extent of structural condensation of the Lck molecule in T cells and created a high-affinity tail mutant based on CLckY-2. Like most SFKs, Lck differs substantially in its C-terminal tail from the optimal SH2-binding sequence phospho-YEEI (37). We exchanged the wild-type tail Y505QPQ by the high-affinity Y505EEI sequence. In agreement with literature, J.CaM1.6 transduced with our mutant construct CLckY-2-YEEI showed a diminished response to CD3 mAb stimulation in respect to Tyr phosphorylation and intracellular calcium flux (Fig. 5, A and C). In correlation with the biochemical data, our high-affinity tail construct showed an increased FRET value of $20.8 \pm 1.9\%$ ($n = 61$ cells; Fig. 5B) and therefore a closed structure.

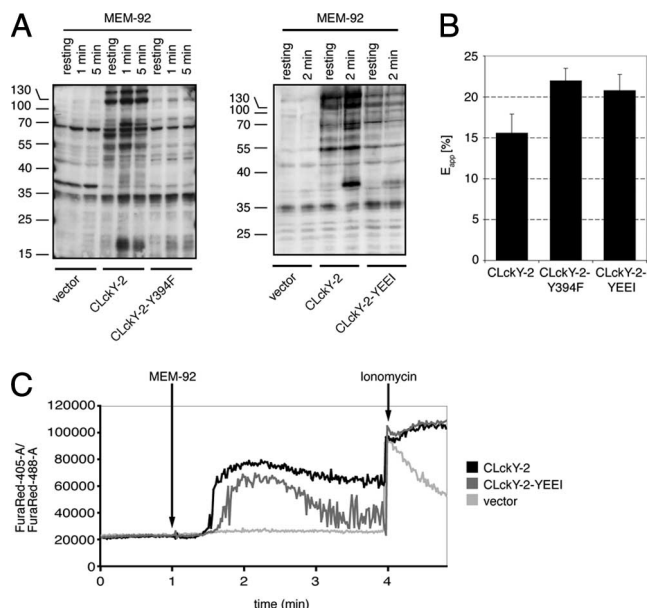


FIGURE 5. Visualization of the crosstalk between regulatory and catalytic domains of Lck. *A*, Western blot analysis of tyrosine phosphorylation. Jurkat J.CaM1.6 cells either transduced with vector, CLckY-2, the Tyr³⁹⁴ to Phe mutant construct CLckY-2-Y394F, or the high-affinity tail mutant CLckY-2-YEEI were stimulated with CD3 mAb MEM-92 for the indicated times. Cells were pelleted and solubilized and the cell lysates were resolved by 12% SDS-PAGE and immunoblotted for tyrosine phosphorylation. *B*, E_{app} of J.CaM1.6 expressing CLckY-2-Y394F and the high-affinity YEEI tail mutant construct CLckY-2-YEEI in comparison to the parental construct CLckY-2. The bar graph depicts mean E_{app} values of cell cohorts analyzed under TIRF illumination 5 min after contact formation with the stimulatory surface; $15.6 \pm 2.3\%$, $n = 291$ for CLckY-2; $22.0 \pm 1.5\%$, $n = 83$ for CLckY-2-Y394F; and $20.8 \pm 1.9\%$, $n = 61$ for CLckY-2-YEEI. *C*, Calcium fluxes as measured by flow cytometry. J.CaM1.6 transduced with empty vector or the Lck constructs CLckY-2 and CLckY-2-YEEI were loaded with the calcium-sensitive dye fura red. Calcium transients in response to CD3 mAb MEM-92 and ionomycin were measured by flow cytometry.

Taken together, our data visualize the dynamic range of Lck condensation in the living cell and show that Lck is kept in an intermediate condensed form via low- to medium-affinity interactions of its regulatory tail sequence with the SH2 domain. Having its safety catch off, Lck can be activated by very subtle mechanisms without the need for changes in regulatory tyrosine phosphorylation and overall structure.

Discussion

Despite years of ongoing SFK research, the space- and time-resolved state of their activities within the cell is not known. This gap exists, because the biochemical methods currently used to address this question are afflicted with an inherent lack of spatiotemporal resolution. The work presented here was aimed at studying and monitoring the conformation-activity relationship of the SFK Lck in living T cells by using genetically encoded FRET biosensors. To gain direct access to the conformational state of Lck, our design was based on the complete kinase backbone. We found the SH2-catalytic domain linker the most vulnerable site for fluorescent protein insertion, as the corresponding construct CLckY-3 was inactive. However, fusion within the SH3-SH2 domain linker was well tolerated, resulting in the biochemically active reporter CLckY-2. This construct revealed a surprisingly high structural flexibility of the

Lck backbone, probably rendered possible by the modular nature of the enzyme.

By TIRF microscopy we found that CLckY-2 exhibited conformation-dependent, intramolecular FRET. We could further demonstrate that CLckY-2 was sensitive to C-terminal tail regulation, as changing the regulatory Tyr⁵⁰⁵ to Phe significantly decreased FRET efficiency and opened the structure of the molecule. However, we did not see a decrease in FRET efficiency of CLckY-2 during T cell stimulation, indicating that the conformation of the biosensor does not change during the activation of T cells.

One explanation for this unexpected result could be that acute dephosphorylation of Tyr⁵⁰⁵ and structural decondensation of Lck are not playing a role during TCR signaling. At least for Jurkat T cells it was described that Tyr³⁹⁴/Tyr⁵⁰⁵ doubly phosphorylated Lck was catalytically active, demonstrating that phosphorylation of the positive regulatory site Tyr³⁹⁴ is dominant over the C-terminal inhibitory Tyr⁵⁰⁵ phosphorylation (38). In line with this finding, the significant FRET increase of our Y394F-mutated biosensor in comparison to the parental construct shows the strong mechanical link between the activation loop and the overall conformation of Lck in living cells. As a result, Lck could remain in a Tyr⁵⁰⁵ phosphorylated but active conformation throughout T cell activation, whereas Tyr³⁹⁴ could act as the master switch for Lck activity and conformation. In accordance with this possibility, we and others (39) have seen that in T cell lines, phosphorylation on Tyr⁵⁰⁵ is not changing during CD3 mAb-mediated stimulation. Even an increase in phosphorylation of Tyr⁵⁰⁵ along with an increase in Lck kinase activity upon CD4 crosslinking was reported (40). This finding is supported by a recent phosphoproteomics study where CD3 crosslinking increased pTyr⁵⁰⁵ 3-fold over control while pTyr³⁹⁴ content was nearly unaffected (41). Our data demonstrating a steady Lck conformation throughout T cell activation indicate that neither modulation of Tyr⁵⁰⁵ nor Tyr³⁹⁴ seem to play a dominant role during TCR triggering.

At the sensitivity and time scale of our measurement methods, we cannot rule out that only a minor part of Lck molecules is being dephosphorylated at a given time point during T cell stimulation. It seems however, that at steady state already the majority of Lck molecules are in a Tyr⁵⁰⁵-dephosphorylated and, thus, primed form, as seen by our own laboratory (data not shown) and by others (39, 42). This substantial, pre-existing pool of primed Lck molecules in the membrane of resting T cells might be sufficient to mediate phosphorylation of the TCR, thus bringing into question the significance of a decrease in pTyr⁵⁰⁵ below the detection limit of our current methods. Based on these assumptions, a further possibility for the regulation of Lck for TCR signaling could be at the level of its localization rather than its molecular modification. Imaging studies suggest an enrichment of Lck at the immunological synapse of T cell APC conjugates (6, 7). At a much smaller scale, TCR microclusters seem to enrich Tyr³⁹⁴-phosphorylated, active Lck (43). Leaving the net balance of phosphorylated Lck forms unchanged, localization-based mechanisms could act as the ignition for TCR signaling.

Finally, when compared with the high-affinity tail and Y505F mutants, the “wild-type” construct CLckY-2 exhibits an intermediate FRET efficiency that can be explained in two ways. First, Lck could exist in an equilibrium state of several subpopulations simultaneously, as proposed by Hermiston and colleagues: 1) open and activated; 2) open and not activated (primed); and 3) closed and not activated (16). Such a heterogeneous mixture of conformational states could give rise to a

characteristic mean FRET efficiency and provide a pool of pre-formed, open, and catalytically active Lck molecules as discussed above.

In a second scenario, Lck could be kept in an intermediate condensed or "half-primed" form via low- to medium-affinity interactions of its regulatory tail sequence with the SH2 domain. Others have already highlighted that most SFKs differ substantially in their C-terminal tails from the optimal SH2-binding sequence phospho-YEEI (37, 44). Low- to medium-affinity tail sequences may have been evolutionary favored to allow SFK activation by higher affinity SH2 or SH3 domain ligands. Indeed, viral proteins such as HIV-1 Nef or HSV Tip have been demonstrated to effectively activate SFKs via high-affinity SH3 interactions (45, 46). T cell-specific adapter protein, TSA_d, is tyrosine phosphorylated upon TCR triggering and stimulates Lck kinase activity by interacting with the Lck SH2 and SH3 domains. Knockout mice for TSA_d exhibit severely impaired proximal TCR signaling due to lower Lck kinase activity (47). Also, the classical costimulatory molecule CD28 carries proline motifs in the cytoplasmic domain that were demonstrated to activate Lck (48) and recruit it to the immunological synapse (49). A similar costimulatory capacity of Lck activity was shown for integrins. Interestingly, soluble ligands to β_1 integrins led to activation of the free Lck pool, while CD4-associated Lck was inhibited by a SHP-1-dependent mechanism (50, 51).

In summary, although our Lck FRET sensor construct is sensitive to Tyr³⁹⁴ and Tyr⁵⁰⁵ phosphorylation/dephosphorylation, we did not detect changes in FRET efficiency during T cell stimulation. Our data therefore do not support acute structural changes induced by modifications in Tyr³⁹⁴ or Tyr⁵⁰⁵ phosphorylation during TCR signaling. One explanation for this finding could be that only a minor population of Lck actively participates in signal transduction. However, we consider that the regulation of signal initiation is governed by novel mechanisms based on the localization of a pool of pre-existing Lck activity in the membrane and on the domain displacement of intramolecular interactions by ligands.

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Disclosures

The authors have no financial conflict of interest.

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