



The effects of membrane compartmentalization of csk on TCR signaling

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ABSTRACT

The TCR signal transduction is initiated by the activation of Src-family kinases (SFK) which phosphorylate Immunoreceptor tyrosine-based activation motifs (ITAM) present in the intracellular parts of the T-cell receptor (TCR) signaling subunits. Numerous data suggest that after stimulation TCR interacts with membrane rafts and thus it gains access to SFK and other important molecules involved in signal transduction. However, the precise mechanism of this process is unclear. One of the key questions is how SFK access TCR and what is the importance of non-raft and membrane raft-associated SFK for the initiation and maintenance of the TCR signaling. To answer this question we targeted a negative regulator of SFK, C-terminal Src kinase (Csk) to membrane rafts, recently described “heavy rafts” or non-raft membrane. Our data show that only Csk targeted into “classical” raft but not to “heavy raft” or non-raft membrane effectively inhibits TCR signaling, demonstrating the critical role of membrane raft-associated SFK in this process.

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1. Introduction

C-terminal Src kinase (Csk) [1] is a key negative regulator of Src-family kinases (SFK), enzymes associated mostly with membrane rafts. Csk phosphorylates tyrosine residues located in the C-terminal tails of Src-family kinases such as Lck [2] which promotes their inhibited (closed) conformation and thus suppresses signaling by various surface receptors, including T-cell receptor (TCR). The inhibitory phosphotyrosine is dephosphorylated by transmembrane phosphatases, most prominently by CD45 [3]. Thus, the proper level of activity of SFK is set by the balance between these two key regulators. While CD45 is a transmembrane protein, most of Csk is localized in the cytoplasm and is recruited to its plasma membrane substrates (SFK) via membrane-associated adaptor proteins. The inhibitory activity of Csk on TCR signaling can be markedly reduced by a mutation (R107K) abrogating the functionality of Csk SH2 domain which is essential for Csk interaction with the membrane-associated adaptor proteins. Fusion of Csk to the myristoylation membrane targeting signal from c-Src was reported to restore the ability of the R107K mutant to inhibit TCR-triggered signals, confirming that Csk recruitment to the plasma membrane is crucial for its function [4].

In T lymphocytes, a significant fraction of SFK is found in membrane rafts. Two of the membrane raft resident proteins, Phosphoprotein

associated with glycolipid-enriched microdomains (PAG) and Lck-interacting membrane protein (LIME), interact with Csk and thus contribute to negative regulation of SFK in this critical compartment [5–8]. However, lymphocytes from PAG and LIME knock-out mice do not show any apparent dysregulation of TCR signaling which would be expected due to the reduced levels of membrane-associated Csk [9–11]. This either indicates that there are additional adaptors bringing Csk to membrane rafts [12–15], or, alternatively, it may also suggest that membrane raft-associated fraction of SFK is not critically important in TCR signal transduction since a significant pool of SFKs can be found outside membrane rafts [16]. Moreover, a study where Src-homology 2 containing phosphatase 1 (SHP-1) was targeted to lipid rafts via a Linker for activation of T cells (LAT)-anchoring sequence showed strong inhibition of LAT phosphorylation and other events in TCR signaling cascade by this construct, but the proximal phases including SFK dependent ITAM phosphorylation and zeta-associated protein of 70 kDa (ZAP-70) activation were unaffected, again casting doubt over the importance of lipid raft pool of SFK [17]. In line with these data also seemed to be the observation that the effects of Csk SH2 domain inactivation can be reversed by the membrane targeting of this construct via c-Src myristoylation sequence [4]. The myristoylation signal from c-Src is not generally believed to target proteins to membrane rafts and thus this observation indicates that perhaps targeting of Csk to these microdomains (as brought about by PAG/LIME) is not necessary for the regulation of the pool of SFK involved in TCR signaling. However, it is also possible that the Src targeting signal allows for membrane raft access, but due to its specific nature and/or lack of palmitoylation its association with membrane rafts may be more transient and difficult to detect by biochemical approaches.

Recently, we described a new type of raft-like microdomains producing upon detergent solubilization “heavy DRM” (“heavy

Abbreviations: DRM, detergent-resistant membrane; SFKs, src-family kinases; LAT, linker for activation of T cells; Csk, C-terminal Src kinase; OFP, orange fluorescent protein; LM, laurylmaltoiside

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Detergent-resistant membranes“) [18]. These microdomains, to be called here for the sake of simplicity (albeit somewhat imprecisely) “heavy rafts”, contain a number of membrane molecules, appear to be similar to the “classical rafts” in yielding large (as judged by Sepharose 4B gel filtration) DRMs resistant to solubilization by polyoxyethylene type detergents, such as Brij-98, but sensitive to laurylmaltoside and to cholesterol extraction. In contrast to classical raft-derived DRMs, the heavy DRMs do not float in density gradient and are more dependent on protein–protein interactions [19]. Targeting of a key T cell signaling molecule, transmembrane adaptor protein LAT, to heavy rafts rescued TCR signaling in LAT deficient cell line, albeit less effectively than targeting into “classical” rafts (where LAT is normally present). This explained a previous puzzling result [20] indicating that targeting of LAT to membrane rafts is actually not as essential as originally thought [21,22].

In fact, there is very little direct functional evidence for the importance of membrane raft targeting of signaling molecules such as SFK or transmembrane adaptor proteins. As far as we are aware, there is only a single report demonstrating the importance of palmitoylation-based Lymphocyte-specific protein tyrosine kinase (Lck) targeting into T cell membrane rafts not only for proper plasma membrane localization but also for full signaling efficiency [23].

Therefore, it was of interest to compare functional effects of targeting of Csk into classical membrane rafts, newly described heavy rafts and non-raft membrane. The results should identify the plasma membrane compartment(s) in which resides the fraction of endogenous SFKs responsible for the initiation of TCR signaling. This approach is complementary to the one used by Kabouridis et al. [23] based on Lck mutant transfectants.

To achieve this we constitutively targeted Csk to the Jurkat T cell membrane by joining Csk with various membrane-targeting motifs. Our data show that signaling through TCR can be completely inhibited by several Csk constructs containing raft targeting motifs but not by constructs targeted to non-raft membrane or to heavy raft-like microdomains. The results further confirm marked functional difference between the classical and heavy rafts and support the concept of essential importance of membrane rafts in initiation of TCR signaling.

2. Materials and methods

2.1. DNA cloning, cells and antibodies

Human Csk cDNA was mutated via fusion PCR to introduce E154A, W47A, R107K mutations and ligated at the C-terminus with Orange fluorescent protein (OFP) [24] using Myc-tag as a spacer, all these constructs were subsequently cloned into pMXs retroviral vector (kindly provided by Dr. A. Cerwenka, DKFZ, Heidelberg, Germany) [25] via BamHI–Sall. Next, we inserted various transmembrane domains (see Fig. 1A for details) at the N-terminal part of Csk-OFP via BamHI. All constructs were verified by DNA sequencing. To prepare inducibly targeted Csk molecules, we obtained all necessary reagents from Ariad Pharmaceuticals (Cambridge, MA; www.ariad.com/regulationkits). LAT TM domain sequence was joined with FRB domain, we used FLAG Tag as a spacer to ensure flexibility of the FRB domain and the construct was cloned into pMSCV vector expressing Thy1 as a surrogate marker from an IRES sequence placed downstream. Next, Csk-OFP was joined with a tandem FKBP domain and cloned into pMXs retroviral vector, the FKBP domain was placed downstream of the Csk-OFP and was separated via 8×Gly linker. All constructs were verified by DNA sequencing. Jurkat cells were from ATCC, and rabbit antiserum to human LAT was kindly provided by Dr. L. Samelson (NIH, Bethesda, MD), rabbit antiserum to human Lck by Dr. A. Veillette (Clinical Research Institute of Montreal, Montreal, Canada), and mAb C305 to Jurkat cell TCR by Dr. A. Weiss (UCSF, San Francisco, CA). Antibodies to CD69 (FITC-labeled; BD Biosciences, San Jose, CA), CD25 and Thy1 (FITC and APC labeled;

eBioscience, San Diego, CA), Phosphotyrosine (4 G10) (Millipore, Billerica, MA), phospho-Lck Tyr505, phospho-Src-family Tyr416 and Myc-tag (Cell Signaling Technology, Danvers, MA) and mouse anti-Lck (Exbio, Prague, Czech Republic) were obtained from the indicated commercial sources. To visualize proteins by Odyssey infra-red scanner (Li-Cor Biosciences, Lincoln, NE), we detected primary antibodies with goat anti-mouse, or goat-anti rabbit secondary antibodies labeled with IR680 or IR800 dyes (Li-Cor Biosciences).

2.2. Retroviral infection and FACS sorting

Retroviruses (RV) were prepared by transfection of Phoenix-Ampho cells (Origene, Rockville, MD) with plasmid DNA using Lipofectamine (Invitrogen, Carlsbad, CA) in six-well plates. RV-containing supernatant was centrifuged to remove debris and then used to spin-infect (1200×g/90 min at room temperature) Jurkat cells in the presence of Polybrene (10 µg/ml, Sigma-Aldrich, St. Louis, MO). Cells were allowed to expand in culture and then sorted using FCAS Vantage cell sorter (BD Biosciences, San Jose, CA) to isolate infected OFP⁺ cells.

2.3. Analysis of phosphorylated proteins

Cells (5×10^7 /ml) were stimulated with C305 Ab (5 µg/ml) at 37 °C, at each time point, 0.1 ml of cell suspension were mixed with equal volume of 2× sample buffer, sonicated and further denatured for 3 min at 95 °C. Phosphorylated proteins were resolved by SDS-PAGE and detected with mouse anti-phosphotyrosine (4G10). To measure the levels of src-family tyrosine 416 and 505 phosphorylation, the lysates were immunoblotted with rabbit anti-phospho-Lck Tyr505, rabbit anti-phospho-Src family Tyr416 and mouse anti-Lck antibodies. The AP21967 treatment was carried out so that cells (5×10^7 /ml) were incubated for indicated time with 0.5 µM AP21967 and then lysed immediately in SDS sample buffer. In the case when we used infrared fluorescence detection using Odyssey scanner, we stained the membrane with mouse anti-Lck Ab and rabbit anti-pY416 or, anti-pY505 antibodies followed by goat anti-mouse IR680 and goat-anti rabbit IR800 secondary antibodies. To quantify signal intensities we exported the intensity values from the Odyssey software and further processed those using Microsoft Excel software (Microsoft, Redmond, WA) to generate the values of phospho-specific signal normalized to total Lck.

2.4. DRM isolation, immunoblotting

To isolate DRM by gel filtration, cells were first fractionated to purify cellular membranes. Cells (10^8) were resuspended in 0.4 ml of ice-cold hypotonic buffer (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM MgCl₂, protease inhibitor mixture), incubated on ice for 15 min and then passed 10× through the 30-gauge needle. The suspension was centrifuged 5 min at 300 ×g, 2 °C to remove nuclei. The ice-cold post-nuclear supernatant was centrifuged 10 min at 25,000 ×g and 2 °C to sediment the membranes. Membranes were then lysed in 0.2 ml 1% Brij-98-containing lysis buffer (polyoxyethylene 20 oleyl ether; Sigma-Aldrich, St. Louis, MO) for 30 min on ice, and spun at 10,000 ×g for 3 min, and 0.1 ml of the lysate was applied at the top of a 1 ml Sepharose 4B column and sequentially washed with 0.1 ml of the lysis buffer; 0.1 ml fractions were collected (all performed at 4 °C) and analyzed by SDS-PAGE/immunoblotting. To isolate DRM by density gradient ultracentrifugation, cells (5×10^7) were solubilized in 0.5 ml of the lysis buffer containing 1% Brij-98 (30 min on ice), then 0.5 ml of ice-cold 80% (wt/vol) sucrose in lysis buffer was added, and placed at the bottom of a 5.2 ml polyallomer centrifuge tube (Beckman Instruments) and carefully overlaid with 1.8 ml of 30%, 0.8 ml 20%, 0.8 ml 10% and 0.7 ml 5% sucrose in lysis buffer (with the detergent) and finally with 0.1 ml of lysis buffer without sucrose.

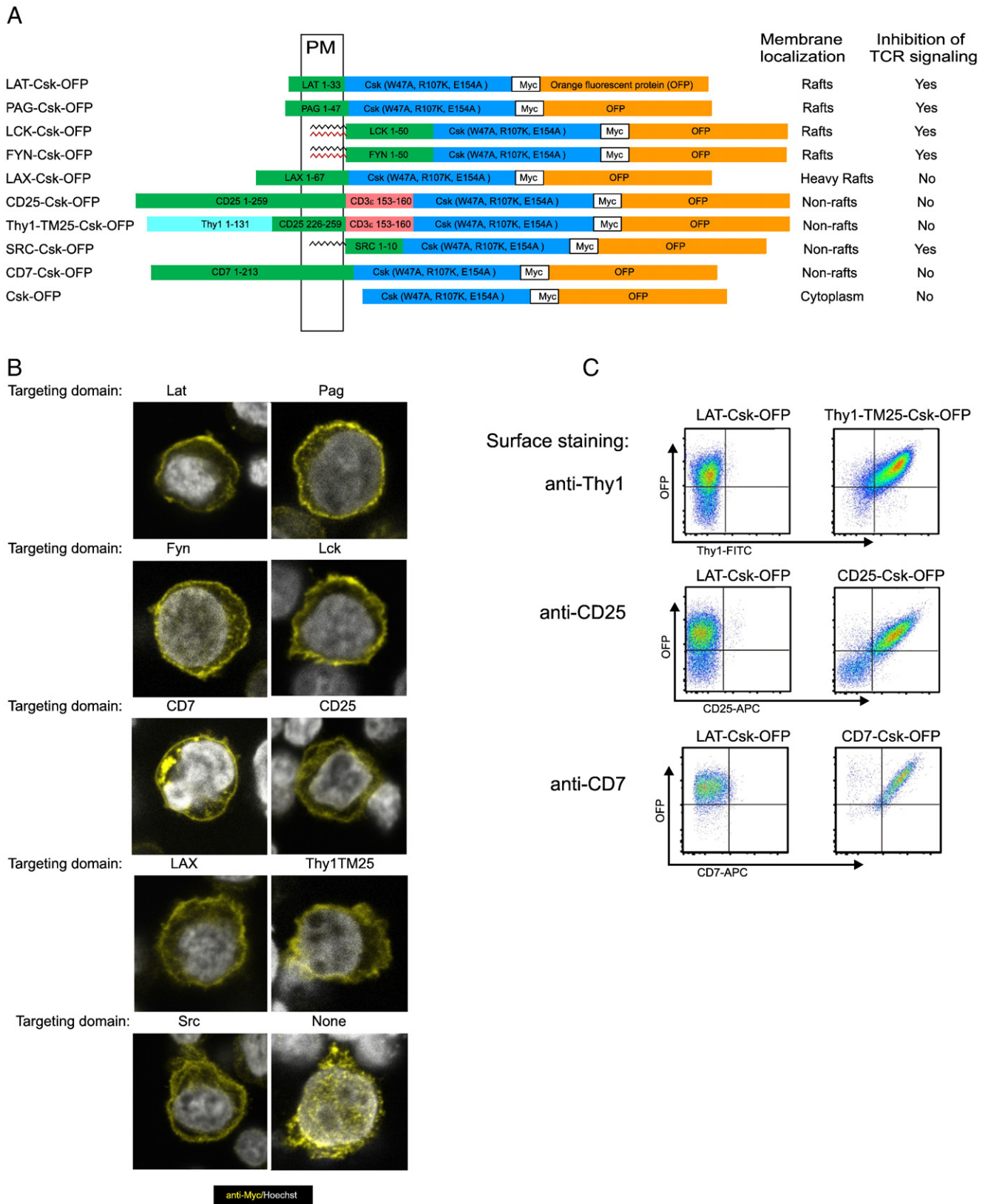


Fig. 1. Constructs scheme and subcellular localization of Csk-OFP constructs. A: The Abbreviations: PM, plasma membrane; OFP, orange fluorescent protein; the numbers in the constructs denote the respective amino acid segments. zigzag – myristoyl, red zigzag – palmitoyl residue. B: Jurkat cells expressing the indicated Csk constructs were fixed, permeabilized and stained with anti-Myc Ab followed by Alexa 488-labeled secondary Ab (yellow), nuclei were visualized with Hoechst (gray). C: Jurkat cells expressing CD25-Csk-OFP, Thy1-TM25-Csk-OFP, CD7-Csk-OFP and LAT-Csk-OFP constructs were surface-stained on ice with FITC-labeled anti-Thy1 Ab, APC-labeled anti-CD25 Ab and anti-CD7. The expression of the indicated Csk-OFP construct was analyzed by cytofluorometry on live cells and is plotted against Thy1, CD25 and CD7.

Centrifugation was carried out at 2 °C in Beckman Optima MAX-E ultracentrifuge, using the MLS50 swing-out rotor (18 h, 50,000 rpm). Nine 0.57 ml fractions were collected from top of the gradient and mixed with SDS sample buffer, and proteins were separated by SDS-PAGE and analyzed by immunoblotting.

2.5. Cell stimulation and cytofluorometry

To measure upregulation of CD69, Jurkat cells were stimulated overnight with C305 antibody (purified, 1 µg/ml), or left unstimulated, cells were then washed with FACS buffer (PBS containing 2% fetal calf serum (FCS) and 0.1% sodium azide) and stained on ice with FITC-labeled anti-CD69 antibody. Alternatively, Raji B cells were loaded with 0.1 µg/ml Staphylococcal enterotoxin E (SEE) and incubated overnight with Jurkat cells at 1:1 ratio. To determine the surface expression of CD25-Csk-OFP and Thy1-TM25-Csk-OFP, live cells were similarly stained with antibodies specific for CD25 and Thy1. To measure calcium response to anti-TCR activation, cells expressing Csk chimeras fused with GFP were first loaded with 5 µM Fura-Red (Invitrogen) in loading buffer (1x Hanks balanced salt solution, 2% FCS, without Ca²⁺, Mg²⁺) for 30 min at 37 °C. After washing, cells were resuspended in loading buffer supplemented with Ca²⁺, Mg²⁺, and kept on ice. Before imaging calcium response, cells were warmed for 10 min at 37 °C and then stimulated with C305 mAb (5 µg/ml). The treatment with AP21967 (0.5 µM) was carried out for five min at 37 °C before stimulation with C305 Ab. The calcium response was detected in a ratiometric setup using 405/480 nm excitation. All flow cytometry measurements were performed using LSRII instrument (BD Biosciences). Analysis of the data was performed using the FlowJo software (Tree Star, Ashland, OR). To measure NFAT activity, cells were first electroporated using a GenePulser electroporator (Bio-Rad, Hercules, CA) with a reporter plasmid expressing luciferase from an NFAT-responsive promoter, the luciferase activity was then measured by adding the OneGlo substrate (Promega, Madison, WI, USA) and the luminescence was assayed with the EnVision reader. The TCR response was calculated as a % of maximal response which was determined by stimulating the same cells with PMA/Ionomycin.

2.6. Microscopy

Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min on ice. Cells were then incubated with anti-Myc antibody followed with Alexa 488-labeled goat anti-mouse IgG secondary antibody (Invitrogen), and nuclei were visualized with Hoechst 33258 dye. Images were acquired on Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. Membrane and raft localization of the various csk constructs

To find out how targeting Csk into different membrane compartments inhibits TCR signaling, we prepared several C-terminally GFP-tagged constructs containing distinct targeting motifs (Fig. 1A). To minimize possible complicating effects of interactions of Csk SH2 and SH3 domains with other proteins (regulatory enzymes, adaptors, substrates), we used constitutively active Csk mutant (E154A), which was further mutated to inactivate the SH2 (R107K mutation) and SH3 (W47A mutation) domains [26]. Some of the constructs were designed for targeting into membrane rafts (Lck-Csk-OFP, Fyn-Csk-OFP, LAT-Csk-OFP, LAT162-Csk-OFP, PAG-Csk-OFP), others to newly described heavy raft-like microdomains [19] (LAX-Csk-OFP) or non-raft membrane (CD25-Csk-OFP, Thy1-TM25-Csk-OFP, Src-Csk-OFP, CD7-Csk-OFP); cytoplasmic Csk-OFP served as a control. These

constructs were cloned into retroviral vector and were subsequently used to create the respective Jurkat cell lines stably expressing these constructs. Then, we verified by confocal microscopy (Fig. 1B) and cytofluorometry (in the cases of constructs possessing an extracellular domain, Fig. 1C) that the constructs were properly expressed at the plasma membrane. To characterize the membrane complexes containing Csk constructs we first performed gel filtration analysis of the membrane lysates on Sepharose 4B in the presence of mild lipid raft-preserving detergent Brij-98. As shown in Fig. 2A, all the raft-targeted constructs (LAT-Csk-OFP, Fyn-Csk-OFP, Lck-Csk-OFP, PAG-Csk-OFP) were indeed present exclusively in large complexes, while the presumably non-raft constructs (CD25-Csk-OFP, Src-Csk-OFP, Thy1-TM25-Csk-OFP, CD7-Csk-OFP) were found mostly in low MW fractions or displayed broader distribution clearly different from lipid raft-targeted constructs. As expected from our previous work, majority of the LAX-Csk-OFP construct was also present in a sort of large DRMs apparently corresponding to the heavy raft-like microdomains [19]. A typical feature of classical lipid rafts, as well as heavy raft-like microdomains, is their sensitivity to another mild detergent laurylmaltoide (LM). The results of the gel filtration experiment which was performed in the presence of 1% LM (Fig. 2B) confirmed, that similarly to classical raft-targeted constructs, complexes containing LAX-Csk-OFP were disrupted in the presence of this detergent. Next, to further confirm proper targeting of our constructs, we performed sucrose density gradient ultracentrifugation in the presence of 1% Brij-98 (Fig. 2C). The data demonstrate that targeting via lipid raft-specific anchors (LAT-, Fyn-, Lck-, PAG-) led to an almost exclusive partitioning of Csk to the low density fractions where the classical lipid rafts are typically present. Importantly, none or negligible amounts of the non-raft targeted Csk constructs could be detected in the low density fractions, including the LAX-Csk-OFP construct which was distributed very similarly to the previously described heavy raft-targeted LAX-LAT-OFP construct. To rule out the possibility that differential distribution of the LAT-Csk-OFP and LAX-Csk-OFP constructs is a result of some general change in properties of lipid rafts in the expressing cell lines, we determined the distribution of the endogenous LAT protein in the cells expressing LAT-Csk-OFP, LAX-Csk-OFP, CD25-Csk-OFP, and parental Jurkat cells and we did not find any significant difference (Fig. 2D). We similarly tested remaining Csk-expressing cell lines (data not shown) and found that LAT protein was similarly distributed in all cell lines tested, confirming, that targeting Csk to lipid rafts does not perturb integrity of these microdomains or the raft partitioning of endogenous LAT protein. Collectively, our fractionation experiments confirmed that the insertions of the different membrane anchors target the Csk constructs into various membrane compartments of different detergent resistance (conventional rafts, heavy raft-like microdomains, non-raft membranes).

3.2. Effects of the raft vs. Non-raft csk constructs on TCR signaling

Next we analyzed the effects of Csk membrane targeting on TCR-mediated signaling. As shown in Fig. 3, the TCR-induced tyrosine phosphorylation of cellular substrates was strongly inhibited in cells expressing LAT-Csk-OFP, but not in those expressing LAX-Csk-OFP or CD25-Csk-OFP. This finding indicates that only Csk targeted into conventional membrane rafts is able to effectively inhibit TCR signaling cascade by inactivating SFK necessary for the earliest steps.

In addition to the effects on tyrosine phosphorylation, we determined the ability of the various Csk constructs to inhibit cytoplasmic calcium elevations and CD69 upregulation following TCR cross-linking. To ensure that we analyzed cells expressing equal levels of the Csk-constructs, we compared cell populations of similar GFP fluorescence intensity. As shown in Fig. 4, the cells expressing raft-targeted LAT-Csk-OFP poorly responded to TCR cross-linking as measured by surface CD69 upregulation (Fig. 4A) and intracellular Ca

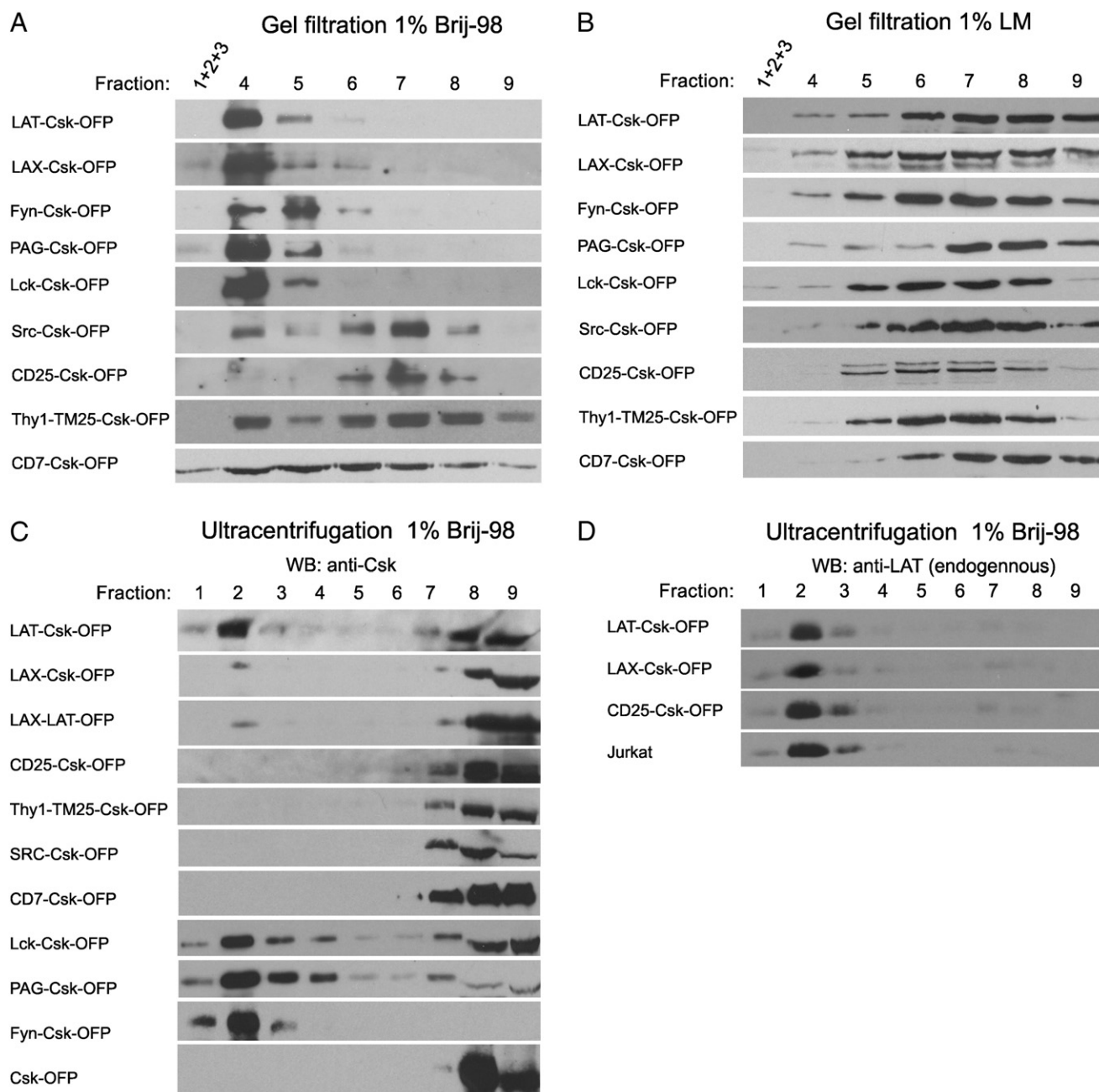


Fig. 2. Association of Csk constructs with (raft-like) detergent-resistant membrane complexes. A,B: Cells were homogenized to isolate cell membranes which were then solubilized in the presence of 1% Brij-98 (A) or 1% LM (B) and subjected to gel filtration on Sepharose 4B, Csk constructs were visualized by immunoblotting using anti-Myc Ab. C: Cells were lysed in the presence of 1% Brij-98 and subjected to sucrose density gradient ultracentrifugation. The LAX-LAT-OFP construct was expressed in J.Cam2 cells as originally described [18]. Constructs were visualized by immunoblotting using anti-Csk antibody. D: Distribution of endogenous LAT protein in the sucrose density gradient fractions prepared from the indicated cell lines.

increase (Fig. 4B). Similar inhibition of signaling was observed also in cells expressing Csk targeted to the plasma membrane via other lipid-raft specific anchors, such as TM domain of the adaptor protein PAG, N-terminal double-acylated motif of kinases Lck and Fyn, and also myristoylated motif of Src. In contrast, in cells expressing Csk tethered to the membrane via non-raft anchors (CD25-Csk-OFP, CD7-Csk-OFP) or the N-terminal motif of LAX (Linker for activation of X cells). TCR signaling was minimally inhibited. The observation that all lipid-raft specific anchors (i.e. those derived from LAT, PAG, Lck, Fyn) are equally efficient suggests that the possible heterogeneity among various conventional membrane raft microdomains is not important, at least for the functional interaction between Csk and the SFK involved in the initiation of TCR signaling. The efficiency of the

N-terminal motif of Src may be explained by its weak and/or transient interaction with lipid rafts as discussed below.

Additionally, we tested the ability of the various Csk constructs to inhibit Jurkat T cell activation following stimulation with SEE-pulsed Raji B cells (Fig. 4C, D). The data in Fig. 4C demonstrate that superantigen-pulsed Raji efficiently induced upregulation of CD69 in Jurkat cells expressing non-raft LAX-Csk and CD25-Csk but we observed minimal activation of cells expressing lipid-raft targeted LAT-Csk and Lck-Csk constructs. Next, we determined the ability of SEE-pulsed Raji cells to activate NFAT transcription factors (Fig. 4D) in cells transfected with NFAT-luciferase reporter DNA plasmid. The data show that lipid raft-targeted Csk efficiently inhibits NFAT-response while non-raft targeted Csk does not. In summary, the presented data

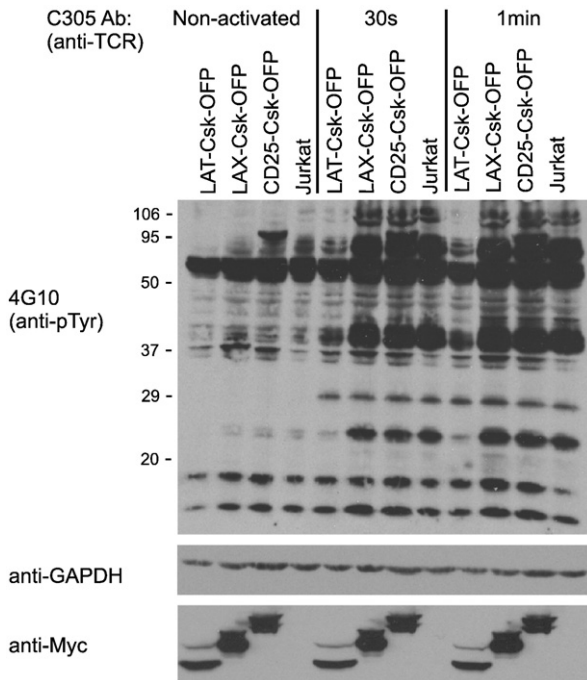


Fig. 3. Inhibition of TCR-induced protein phosphorylation by raft-targeted Csk. Jurkat cells expressing LAT-Csk-OFP, LAX-Csk-OFP, CD25-Csk-OFP and uninfected Jurkat were activated with C305 Ab (5 μ g/ml), or non-activated controls were lysed after indicated time in SDS loading buffer, and phosphorylated proteins were separated by SDS PAGE/immunoblotting and visualized with 4 G10 anti-pTyr Ab. Anti-GAPDH staining demonstrates equal loading, anti-Myc staining shows the levels of expression of each Csk construct.

demonstrate that lipid raft-targeted Csk constructs inhibit multiple downstream events such as calcium response, CD69 upregulation, tyrosine phosphorylation and NFAT activity following TCR activation while non-raft Csk constructs fail to inhibit these events.

To demonstrate that the inhibition of TCR signaling is dependent on Csk kinase activity, we generated another mutant, a kinase-dead variant of LAT-Csk-OFP which contains inactivating K222R mutation in the kinase domain in addition to the W47A, R107K, E154K mutations introduced previously. The data in Fig. 5A show that Jurkat cells expressing very high amounts of the kinase-dead LAT-Csk-OFP construct efficiently upregulated CD69 following TCR stimulation while cells expressing equally high amounts of the constitutively active form of LAT-Csk-OFP construct completely failed to upregulate CD69.

Next, we wanted to verify that the non-inhibiting constructs possess comparable kinase activity to the raft targeted ones. To test this, we co-transfected LAT-Csk-OFP, LAX-Csk-OFP, and CD25-Csk-OFP constructs with kinase-inactive Lck as a substrate into 293 cells and determined the level of tyrosine 505 phosphorylation in the lysates by immunoblotting with pY505 specific antibody. In our experience in this type of transient overexpression experiment the concentration of the individual components is high enough to overcome the need for specific targeting or compartmentalization [27]. The results show comparable increase in Y505 phosphorylation in all cells expressing active Csk irrespective of the type of the targeting signal Fig. 5B, proving that the lack of inhibition of TCR

signaling in cells expressing LAX-Csk-OFP and CD25-Csk-OFP is not due to insufficient catalytic activity of the Csk kinase domain.

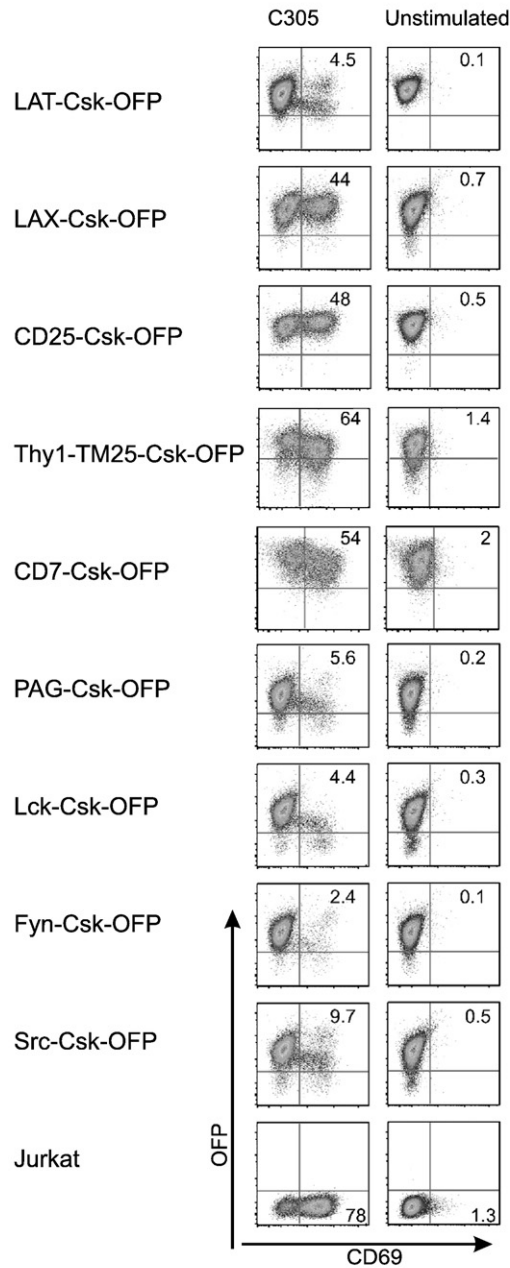
It is well established that Csk inhibits SFK by promoting their closed conformation via phosphorylation of their C-terminal tyrosine (Y505 in human Lck). To test if the same mechanism is responsible for the inhibition of TCR signaling in the cell lines used throughout our study, we measured tyrosine phosphorylation of both inhibitory tyrosine (Y505 in Lck) as well as activation loop tyrosine (Y394 in Lck, Y416 in Src) by immunoblotting with site-directed phospho-specific antibodies. Surprisingly, we have not seen any differences in the phosphorylation of the SFK inhibitory tyrosine among the cell lines tested while there was a clear reduction in phosphorylation of the activation loop tyrosine in the LAT-CSK-OFP expressing cells as compared to non-raft or heavy raft constructs (LAX-Csk-OFP, CD25-Csk-OFP) (Fig. 6A). In order to better understand this surprising result, we adopted an experimental system which enabled us to rapidly recruit Csk to lipid rafts. It is based on inducible heterodimerization of FKBP and FRB domains via a rapamycin analog AP21967 (Ariad Pharmaceuticals). We have generated a Jurkat cell line stably expressing FRB domain fused with LAT transmembrane domain. This cell line was then transduced with retroviruses expressing Csk-OFP fused at the C-terminus with a tandem FKBP domain, followed by FACS purification of transduced cells. The data in Fig. 6B show that the addition of AP21967 led to the Csk membrane recruitment. Consequently, 5 min pretreatment of cells with AP21967 significantly inhibited calcium response following C305 stimulation (Fig. 6C). The duration of AP21967 pretreatment had no measurable effect on the inhibition of TCR signaling, moreover, the calcium response could be rapidly downregulated when AP21967 was added to the cells already activated with anti-TCR antibody at the peak of calcium response (data not shown). Thus, this experimental system allowed us to analyze the phosphorylation status of Lck following rapid recruitment of Csk to the membrane. The data in Fig. 6D show that following addition of AP21967, the phosphorylation level of tyrosine 505 rapidly increases but does not remain stable and within 30 min after addition of AP21967 decreases to the levels present before addition of AP21967. At the same time, the level of phosphorylation of the activatory tyrosine 416 rapidly decreases and remains low. These data suggest that Csk membrane recruitment probably activates regulatory feedback loops reducing overall SFK phosphorylation status, however, these mechanisms do not seem to be able to revert the functional inhibition imposed by Csk, since the ratio between inhibitory tyrosine and activation loop phosphorylation remains largely preserved during the process.

4. Discussion

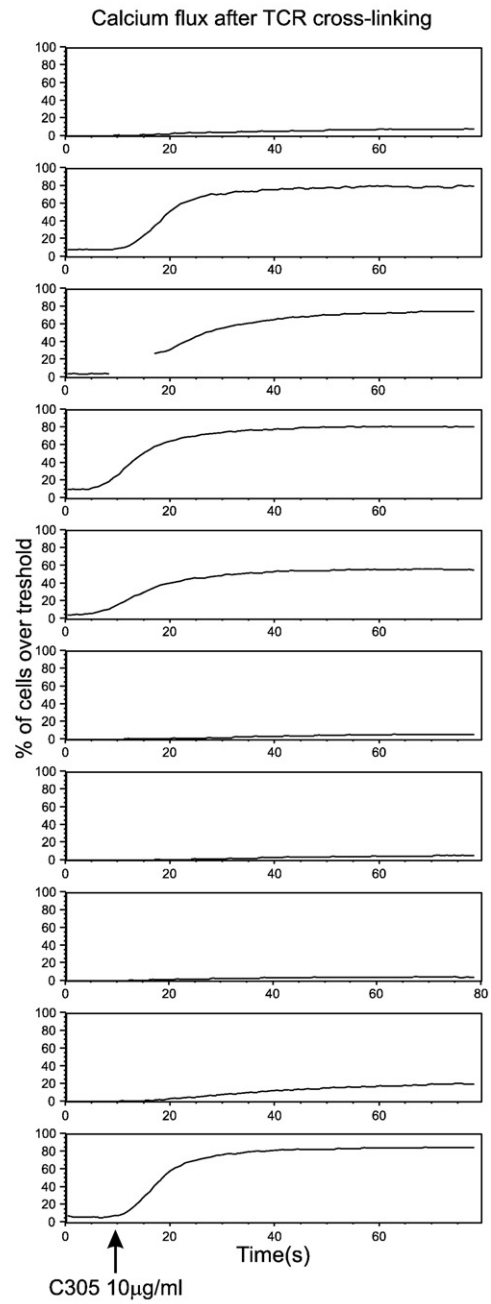
Our present study employed differentially targeted Csk constructs as tools to specifically inhibit Src-family kinases in different plasma membrane subcompartments. We prepared a series of Csk constructs tethered to plasma membrane by means of several motifs known to target into different lipid microenvironments such as membrane rafts (motifs derived from N-terminal sequences of typical raft proteins such as Lck, Fyn, LAT, PAG), recently described heavy raft-like microdomains (targeting sequence from transmembrane adaptor LAX) [19] and non-raft membrane (constructs containing transmembrane domain from CD25). Since SH2 and SH3 domains are involved in Csk targeting, we inactivated SH2 and SH3 domains in our constructs by point mutations to prevent these domains from

Fig. 4. Activation responses of Jurkat cells expressing various Csk constructs. A: The indicated Jurkat cells were stimulated with C305 antibody (5 μ g/ml) and dynamics of their cytoplasmic calcium response was measured, as described in Materials and methods. Cells were gated on OFF⁺ similarly as in B. B: The indicated Jurkat cell transfectants were stimulated overnight with soluble C305 antibody (1 μ g/ml), or left unstimulated. Surface expression of the activation marker CD69 was then measured by cytofluorometry. The numbers show the percentages of the CD69-positive cells out of total OFF⁺ cells. Control Jurkat cells which do not express any Csk construct are shown in the bottom panel. C: Jurkat cells were incubated overnight at 1:1 ratio with Raji B cells either pulsed with SEE (0.1 μ g/ml) or non-pulsed, the response was then determined by staining with anti-CD69 Ab. The values obtained after stimulation with unpulsed RAJI cells were subtracted from the values obtained after stimulation with SEE-pulsed RAJI cells. D: Jurkat cells were electroporated with NFAT-luciferase reporter plasmid and then stimulated similarly as in C, the maximum response was determined after stimulation with PMA/Ionomycin.

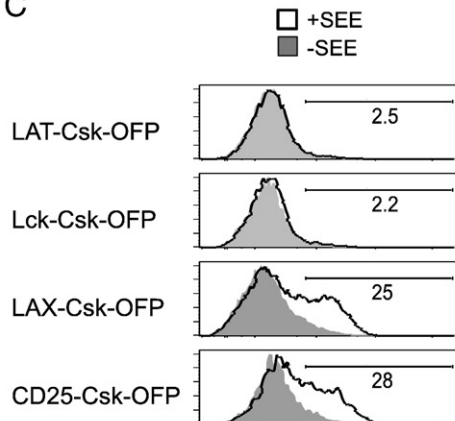
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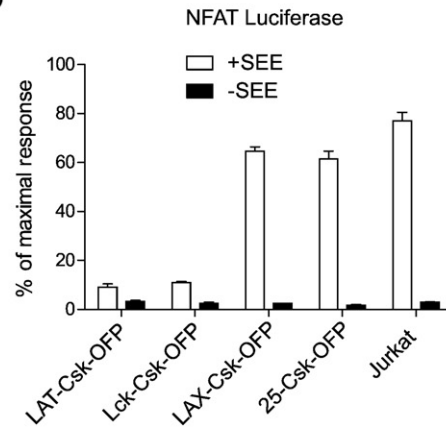
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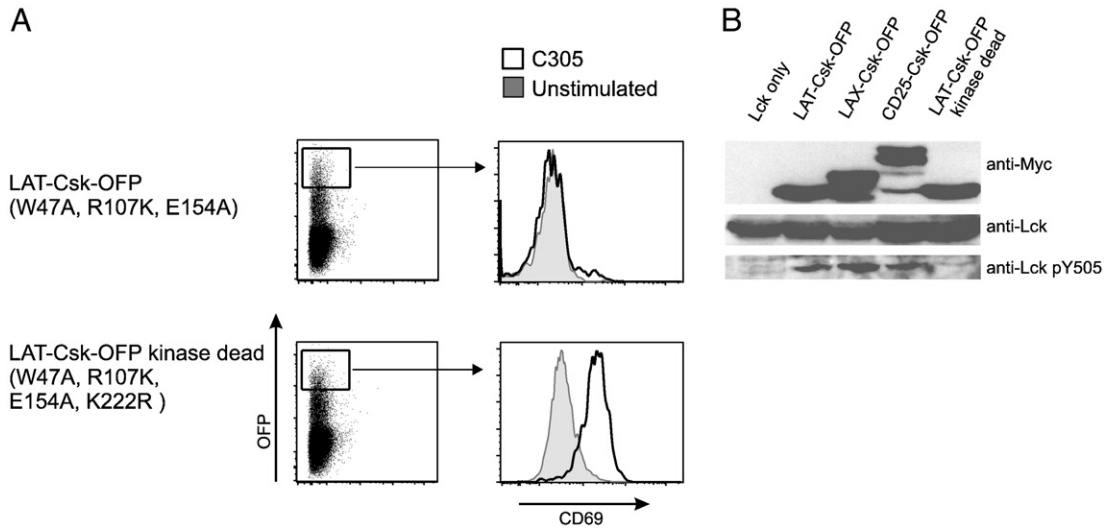


Fig. 5. Kinase activity of Csk. A: Jurkat cells expressing constitutively active LAT-Csk-OFP or kinase-dead LAT-Csk-OFP constructs were stimulated with anti-TCR antibody C305 and then stained for the expression of activation marker CD69. B: 293 cells were co-transfected with kinase-inactive Lck mutant and indicated Csk constructs, or with Lck only. The activity of Csk was determined by immunoblotting with pY505 specific antibody. Anti-Myc staining shows the level of expression of Csk constructs, anti-Lck shows the level of expression of the substrate.

overriding interactions mediated by the N-terminal motifs. However, this approach created a potential problem, since binding of ligands to the SH2/SH3 module is also important for activation of the Csk kinase

domain [26]. This is the reason why we also introduced an activating mutation (E154A) to render the kinase sufficiently active even in the absence of SH2/SH3 interactions [26].

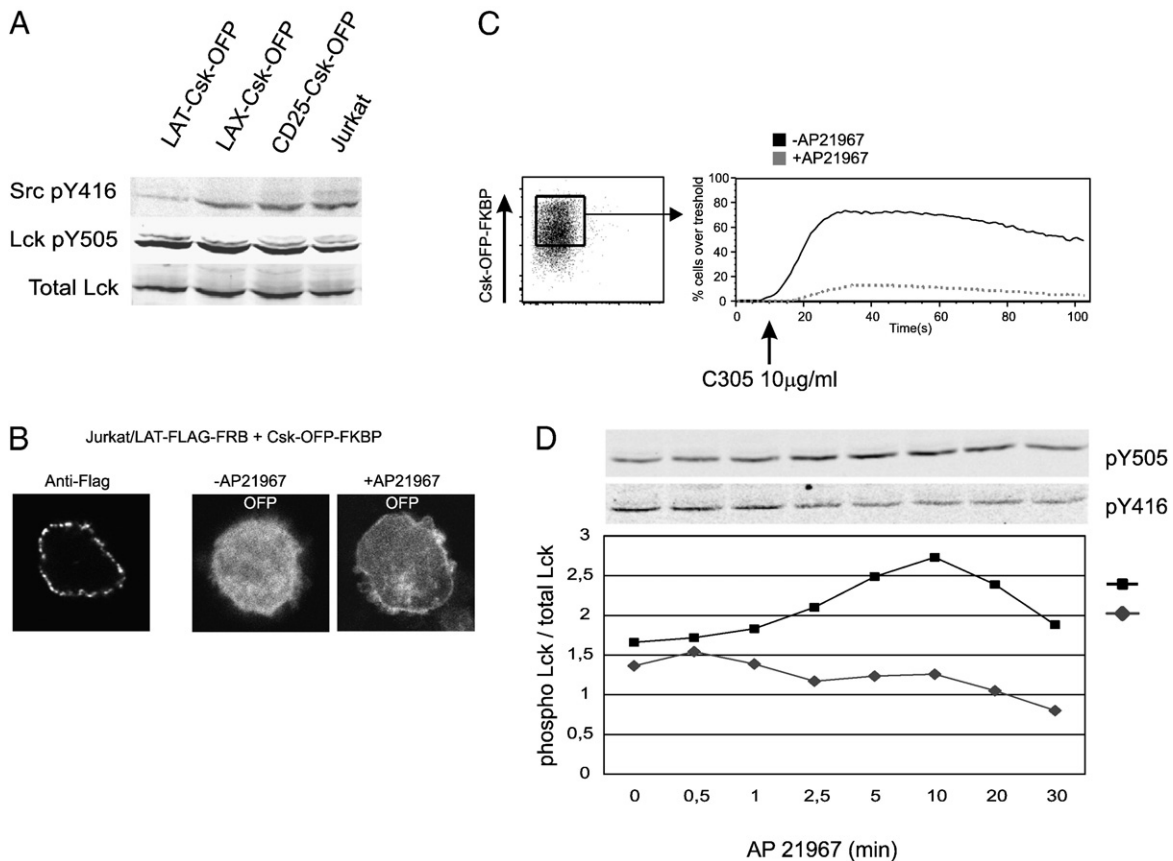


Fig. 6. Phosphorylation of SFK in Jurkat cells expressing various Csk constructs. A: Jurkat cells expressing the indicated constructs were lysed and subjected to immunoblotting. The levels of pY505 and pY416 were determined by immunoblotting with phospho-specific antibodies followed by quantification with Odyssey infra-red scanner. The data represent one typical experiment out of three performed which all gave similar results. B: Confocal image of Jurkat cells expressing LAT-FLAG-FRB and CSK-OFP-FKBP stained with anti-Flag antibody (left panel). The same cells were treated with AP21967 (0.5 µM) for 30 min and localization of Csk-OFP-FKBP was then analyzed by confocal microscopy. C: The same cells were pre-treated with AP21967 (0.5 µM) for 5 min and then the calcium response following activation with anti-TCR antibody C305 was measured. D: Jurkat cells expressing LAT-FLAG-FRB and Csk-OFP-FKBP were treated for the indicated time intervals with AP21967 (0.5 µM) and analyzed as described in A. The data represent one typical experiment out of three performed which all gave similar results.

Our approach allowed us to compare relative importance of the pools of endogenous SFK presumably present in different plasma membrane subcompartments (classical raft, heavy raft, non-raft membrane). Importantly, this approach is based on functional readouts in intact cells, not on any detergent solubilization products, and is directed to endogenous SFK. The most efficient inhibition of TCR signaling was achieved with Csk constructs targeted via anchors derived from different membrane raft-associated molecules, while none of the other constructs (with the exception of Src-Csk-OPF as discussed later) were able to inhibit signaling as efficiently. This is an important and highly relevant finding because of continuing discussions about the nature or the very existence of membrane rafts [28–30]. It also identifies lipid rafts as a location where the most efficient regulation of SFK can be achieved and warrants future research on membrane targeting of Csk via lipid raft resident proteins such as PAG and LIME which are in a very good position to exert such regulation. This implies a transient nature of PAG/LIME Csk interaction or an involvement of regulatory feedback loops that our constructs could not elicit.

Our results are consistent with the idea of membrane rafts as separated areas of the plasma membrane accessible only to a restricted set of proteins possessing appropriate targeting motif. Strikingly, all the membrane raft targeted Csk constructs exerted the same level of inhibition independent of whether this motif was derived from Lck, Fyn, LAT or PAG, suggesting that functional heterogeneity within the membrane raft compartment is limited and that perhaps any raft-targeted protein can in principle access any of these structures. Recent data suggest that membrane rafts are very small and dynamic structures containing probably very few protein molecules [31,32]. Thus, the number of protein molecules per raft is probably substantially lower than the overall number of protein species accommodated in these structures globally, which implies that at any given time point there will be a substantial heterogeneity in the protein composition of these microdomains. However, since there may be rapid exchange of proteins among individual rafts, this heterogeneity might not be apparent on the time scale of our experiments. Furthermore, it is likely that any rafts (of the classical type, yielding upon detergent solubilization buoyant DRMs), regardless of their protein composition, may interact, due to their favorable lipid composition, with TCR.

Surprisingly, we observed strong inhibition of TCR signaling when the Csk construct targeted via N-terminal membrane targeting motif of Src was used. We saw a relatively small but clearly detectable fraction of Src-Csk-OPF in DRM-like large complexes sensitive to laurylmaltoide extraction, suggesting that this construct may have an access to membrane rafts (Fig. 2). However, we failed to detect any Src-Csk-OPF in low density fractions after sucrose density ultracentrifugation. Data published on Src association with membrane rafts have been rather controversial so far, with several studies supporting this association and others failing to detect significant Src presence in these microdomains [33–36]. The presence of Src in membrane rafts seemed to depend on its kinase activity and thus possibly on the interaction of Src SH2 domain with some membrane raft resident substrate such as PAG [37,38]. These data suggest that Src is not excluded from membrane rafts, however, this interaction is probably unstable and/or highly dynamic and its biochemical detection may require stabilization by protein-protein interactions. If this is the case, it may be difficult to determine how large fraction of Src-Csk-OPF is present in membrane rafts *in vivo* since this interaction may be prone to disruption during the detergent extraction and lengthy fractionation procedure, which is especially long when sucrose density gradient centrifugation is employed. These data also suggest that widely used non-raft specific membrane probes such as GFP anchored to the membrane via N-terminal part of Src kinase [39] may in fact be localized at least partially to the native membrane rafts.

Recently we described a novel type of raft-like microdomains, detected as heavy DRMs, apparently also capable of certain functional

interaction with TCR signaling [19]. Targeting to these microdomains can be achieved via N-terminal membrane-anchoring segment of transmembrane adaptor LAX. The present data confirm and extend our previous results on the functional difference between classical rafts and the newly described heavy raft-like microdomains [19]. We and others have shown that the LAX-LAT fusion protein targeted to these microdomains is able to support TCR signal transduction albeit less efficiently than LAT targeted to classical rafts [19,40]. In contrast, the LAX-Csk construct (targeted to heavy rafts) used in this study was not able to inhibit TCR signaling at all. By targeting the inhibitory (Csk) rather than activatory (LAT) molecule to this compartment we created an inverse situation to the previously used setting [19]. Our present data indicate that heavy raft-like microdomains and non-raft membrane most likely do not contain any pool of SFK that is critical for initiation of TCR signal transduction.

Notable observations were made when we analyzed the mechanism of how membrane-targeted Csk regulates SFK. The major effect we observed after the long term expression of lipid raft-targeted Csk was reduced phosphorylation of the tyrosine in the activation loop of the SFK catalytic domain. Since this tyrosine is considered to be an autophosphorylation site, reduced SFK activity should logically result in reduced phosphorylation of this tyrosine. On the other hand it is harder to explain why there was no change in the phosphorylation of the C-terminal tyrosine which is the direct substrate of Csk. To better understand this paradox, we employed a dimerizer system allowing for rapid Csk recruitment to the plasma membrane, which helped us to follow the dynamics of this process. We observed rapid initial increase in the SFK inhibitory tyrosine phosphorylation soon after Csk recruitment to lipid rafts. Phosphorylation peaked at 10 min and then it returned back to the initial levels, while phosphorylation of the activating tyrosine gradually declined throughout the experiment. This observation suggested that Csk recruitment to lipid rafts likely activates feedback mechanisms counterbalancing its inhibitory effect. The reduction in SFK activity may for example result in dephosphorylation of proteins recruiting endogenous Csk such as PAG, LIME or Dok-family adaptors, which are all substrates of SFK [5,7,41]. An alternative possibility is an active process involving recruitment of a phosphatase to dephosphorylate SFK tyrosines. However, regardless of the mechanism, such regulatory feedback is not able to overcome the inhibition imposed by the lipid raft-targeted Csk constructs.

Our present data are certainly compatible with the results published recently by Lillemeier et al. [42] showing that after TCR stimulation LAT complexes (i.e. membrane rafts) get into a close contact with TCR microdomains but do not fuse with each other.

Although our data confirm that membrane raft associated SFKs play a critical role in TCR signaling, they do not directly address the function of membrane rafts in this process. A widely discussed possibility is that membrane rafts serve as a vehicle for translocation of several critical signaling molecules to the TCR. However, at least for LAT and Lck it has been recently shown that their cytoplasmic domains are responsible for targeting to TCR microclusters apparently independently of their presence in the membrane rafts [43]. Hence, it seems that instead of being directly involved in the translocation process, membrane rafts may be rather passively transported to the proximity of TCR by the proteins they contain and may perhaps influence the signaling by changing the protein and/or lipid environment in the proximity of activated TCR. This hypothesis is compatible with our present data, however, our model system cannot be used to directly prove/disprove it. It simply shows, that only lipid raft targeted Csk can efficiently inhibit SFKs in intact Jurkat cells, demonstrating specific segregation of SFKs to membrane rafts *in vivo*. This is a novel type of evidence for the existence of membrane rafts as functional units in the plasma membrane of living cells, obtained without any biochemical manipulations often criticized for their proneness to artifact induction.

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