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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.

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 phosporylates 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 DRMs" ("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, laurylmaltoside

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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 flotate 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–SalI. 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, Karlsbad, 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. Loius, MO). Cells were allowed to expand in culture and then were 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 $MgCl_2$, 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.