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Release from Tonic Inhibition of T Cell Activation through Transient Displacement of C-terminal Src Kinase (Csk) from Lipid Rafts*

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Knut Martin Torgersen‡§¶, Torkel Vang‡¶‖, Hilde Abrahamsen‡, Sheraz Yaqub‡, Václav Hořejší, Burkhart Schraven‡‡, Bent Rolstad§, Tomas Mustelin§§¶¶, and Kjetil Taskén‡‖‖**

*From the Departments of ‡Medical Biochemistry and §Anatomy, Institute of Basic Medical Sciences, University of Oslo, Box 1112, Blindern, N-0317 Oslo, Norway, the **Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 14220 Prague, Czech Republic, the ‡‡Immunomodulation Laboratory of the Institute for Immunology, Ruprecht-Karls University Heidelberg, 69120 Heidelberg, and the Institute of Immunology, Otto-von-Guericke-University, 39120 Magdeburg, Germany, and the §§La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037*

In resting peripheral T cells, Csk is constitutively present in lipid rafts through an interaction with the Csk SH2-binding protein, PAG, also known as Cbp. Upon triggering of the T cell antigen receptor (TCR), PAG/Cbp is rapidly dephosphorylated leading to dissociation of Csk from lipid rafts. However, tyrosine phosphorylation of PAG/Cbp resumes after 3–5 min, at which time Csk reassociates with the rafts. Cells overexpressing a mutant Csk that lacks the catalytic domain, but displaces endogenous Csk from lipid rafts, have elevated basal levels of TCR-ζ-chain phosphorylation and spontaneous activation of an NFAT-AP1 reporter from the proximal interleukin-2 promoter as well as stronger and more sustained responses to TCR triggering than controls. We suggest that a transient release from Csk-mediated inhibition by displacement of Csk from lipid rafts is important for normal T cell activation.

Activation of the Src family kinases Lck and Fyn after engagement of the T cell antigen receptor is an initiating event in T cell activation and leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs)[1] within the TCR complex (1). The subsequent recruitment of the tandem SH2 domain containing tyrosine kinase ZAP-70 to phosphorylated ITAMs generates an activated immune receptor signaling complex that is able to initiate downstream events leading to a functional T cell response (2, 3). The control and fine-tuning of the proximal signaling is not only essential for an effective T cell response to antigen, but also for avoiding exaggerated T cell activation and autoimmunity. Thus a balance must be maintained to avoid hypo- as well as hyper-reactivity and immunopathology. The TCR signaling machinery appears to be controlled by setting a threshold for activation to avoid too easy triggering. Suppression of the catalytic activity of Lck and Fyn by phosphorylation of a C-terminal residue (Tyr^{505} in Lck, Tyr^{528} in Fyn^T) by the C-terminal Src kinase, Csk, appears to be an important means of negative regulation of TCR signaling (4–6). Complexed with Csk via binding to its SH3 domain is also a protein tyrosine phosphatase, PEP, that dephosphorylates the activating phosphorylation site (Tyr^{394} in Lck, Tyr^{416} in Fyn^T) (7, 8). Recent discoveries indicate that the assembly of TCR signaling complexes occurs in specific membrane subdomains with high cholesterol and glycolipid contents, called glycosphingolipid-enriched microdomains or lipid rafts (9–11). Key components, including Lck and LAT, are targeted to rafts by virtue of their lipid modifications, whereas other proteins such as the ζ-chain can localize via interaction with raft components only after TCR engagement (11–13). Lipid rafts serve to concentrate and promote specific protein-protein interactions and the tyrosine phosphorylation of signaling intermediates by the Src family kinases during the proximal phases of immunoreceptor signaling via the TCR as well as via the B cell and Fc receptors (14–16).

The ubiquitously expressed, cytosolic Csk tyrosine kinase is critical for suppression of Src-like kinases and necessary for normal thymic development of T cells (17–19). Disruption of the Csk gene leads to unregulated Src kinase activity and early embryonic lethality (17, 18). Moreover, loss of Csk in thymocytes uncouples thymocyte development from control by the TCR (19). Csk consists of an SH3 domain, an SH2 domain and a kinase domain similar to the Src kinases, but lacks the C-terminal regulatory tyrosine as well as the N-terminal lipid modification sites that targets Src kinases to lipid rafts (20). Thus, although Csk has been shown to move to sites of Src activity in fibroblasts (21), it has for some time been unclear how the cytoplasmic Csk can effectively regulate the lipid raft-associated Src kinases and whether this inhibition by Csk is temporarily released upon T cell activation. The recent identification of a lipid raft-associated transmembrane adaptor protein, phosphoprotein-associated with glycosphingolipid-enriched microdomains (PAG) or Csk binding protein (Cbp) with

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¶ Both authors contributed equally.
‖ Fellow of the Norwegian Cancer Society.

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‖‖ To whom correspondence and reprint requests should be addressed: Dept. of Medical Biochemistry, Institute of Basal Medical Sciences, University of Oslo, P. O. Box 1112, N-0317 Oslo, Norway. E-mail: kjetil.tasken@basalmed.uio.no.

[1] The abbreviations used are: ITAMs, immunoreceptor tyrosine-based activation motifs; LAT, linker for activation of T cells; Csk, C-terminal Src kinase; Cbp, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched membrane domains; HA, hemagglutinin epitope; TCR, T cell antigen receptor; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; IL, interleukin; PLC, phospholipase C; wt, wild type; PTK, protein-tyrosine kinase; PAGE, polyacrylamide gel electrophoresis.

the capacity to bind Csk provides a means for localizing Csk to the proximity of its substrates (22, 23). It was shown that Csk is associated with lipid rafts in normal T cells via PAG/Cbp and that Csk dissociates from the rafts concomitantly with TCR-mediated dephosphorylation of PAG/Cbp (22). Here, we extend these findings and show that the raft-associated Csk present in resting T cells is targeted via interaction of the Csk SH2 domain with PAG/Cbp. Furthermore, rapid Csk reassociation with lipid rafts coincides with termination of proximal TCR signaling. In addition, overexpression of a catalytically inactive mutant Csk that displaces endogenous Csk from lipid rafts leads to both constitutive and sustained activation. Thus, Csk acts as a gatekeeper that must be temporarily sent off duty for efficient TCR signaling to occur.

EXPERIMENTAL PROCEDURES

Cell Culture, Stimulation, and Transfection—The human leukemic T cell line Jurkat TAg, a derivative of the Jurkat cell line stably transfected with the SV40 large T antigen (24), and the Lck-deficient JCaM1 cell line (25) were kept in logarithmic growth in RPMI medium supplemented with 10% fetal calf serum and antibiotics. Human peripheral blood T cells were purified from normal donors by negative selection (26). T cells were activated by addition of 5 μg/ml anti-CD3 ϵ mAb OKT-3 or by pervanadate treatment (0.01% H_20_2 in 100 μM Na_3VO_4). For transfections, cells (2×10^7) in 0.4 ml of Opti-MEM were mixed with 2–80 μg of each DNA construct in electroporation cuvettes with 0.4-cm electrode gap (Bio-Rad) and subjected to an electric field of 250 V/cm with 960-microfarad capacitance. The cells were expanded in complete medium and harvested after 20 h.

Lipid Raft Purification—Isolation of lipid rafts or glycoprotein-enriched membrane microdomains was performed as described in detail elsewhere (11). Briefly, cells were homogenized in 1 ml of an ice-cold lysis buffer (25 mM MES, pH 6.5, 100 mM NaCl, 5 mM EDTA, 1.0% Triton X-100 with 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, and 50 mM sodium fluoride) by 10 pestle strokes in a Dounce homogenizer, loaded at the bottom of a 40 to 5% sucrose gradient, and centrifuged at 200,000 $\times$ *g* for 20 h. Fractions (0.4 ml) were collected from the top.

Immunoprecipitation and Immunoaffinity Purification—For immunoprecipitation, cells (5×10^7) were disrupted in precipitation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 60 mM *n*-octyl-β-D-glucoside, with 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, and 50 mM sodium fluoride). When stimulated with OKT-3, cell lysates were precleared by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at 4 °C and subjected to immunoprecipitation with anti-HA mAb (Babco, Richmond, CA), anti-Csk antibody (Santa Cruz, catalog number SC-286) or anti-PAG mAb (MEM-250) (22). After overnight incubation at 4 °C, protein A/G-Sepharose was added and the incubation continued for 1 h. Immune complexes were washed three times in lysis buffer and subjected to Western blot analysis.

Immunoblot Analysis—Detection of phosphotyrosine by anti-Tyr(P) mAb (4G10, Upstate Biotechnology, Lake Placid, NY), and immunoblotting with anti-Csk, anti-PAG, anti-LAT, anti-PLC-γ1, anti-Grb2, anti-HA, and anti-ζ antibodies was as before (27, 28) except that anti-Csk antibody and anti-ζ antibody from Santa Cruz Biotechnology Inc. (SC-286 and SC-1239, respectively) were used. The anti-PAG antibody (and MEM-255) and the far-Western protocol were described previously (22).

Generation of Csk Constructs and Recombinant Protein—The gene encoding human Csk (29) was subcloned into the expression vector pEF-BOS/HA at *Nhe*I-*Xba*I sites. Vectors encoding HA-Csk-SH3-SH2 and GST-Csk-SH3-SH2 were generated by stop mutations in amino acid 175 using full-length Csk in the respective vectors as template and using a site-directed mutagenesis kit (QuikChange, Stratagene, La Jolla, CA). Subsequently, HA-Csk-SH3-SH2(R107K), HA-Csk-SH3-SH2(S109C), and HA-Csk-SH3(W47A)-SH2 were made in a second round of mutagenesis to eliminate SH2 and SH3 binding capacity, respectively (30–32), whereas a full-length, kinase-dead Csk, HA-Csk(K222R) (6), was generated by mutagenesis of the wild type HA-Csk. GST-Csk-SH3-SH2 was purified as described previously (29). Mutants were verified by sequencing. The FLAG-PAG construct was described previously (22).

Csk Affinity Chromatography—For purification of Csk-associated proteins, purified peripheral T cells (5×10^8) were disrupted in precipitation buffer (as described above including 60 mM *n*-β-octyl-D-glucoside to disrupt rafts) and passed over a column where GST-Csk-SH3-SH2 was bound to glutathione-agarose, followed by washing with 50 bead volumes and elution with 50 mM phosphotyrosine in the same buffer.

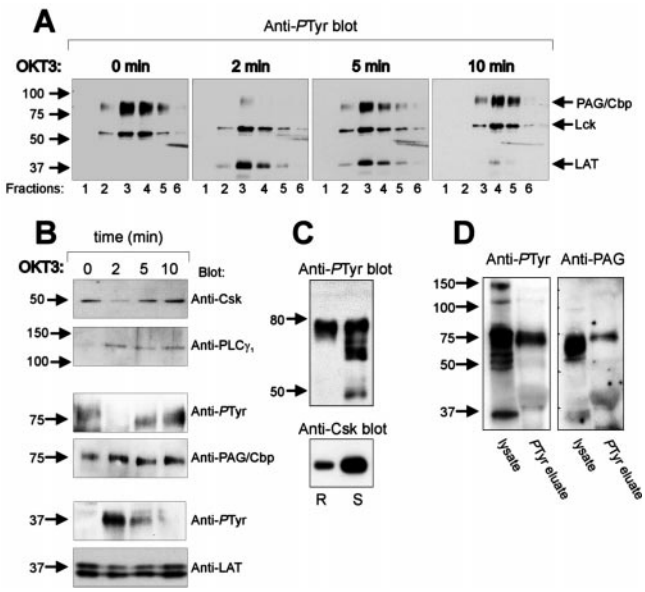

FIG. 1. **Csk is detached from lipid rafts upon T cell activation, but rapidly reassociates.** *A*, purified human T cells were incubated with anti-CD3 antibodies (OKT-3) on ice for 30 min, washed twice, and then the TCR was cross-ligated by $F(ab')_2$ fragments for the indicated time periods (0–10 min) at 37 °C. Thereafter, standard lipid raft fractionation of T cell lysates from all time points was performed. Of the 12 fractions obtained from each separation, anti-phosphotyrosine immunoblots of the upper six fractions containing the rafts are shown. Longer exposures of the same blots also showed Vav, Slp-76, Zap-70, and other proteins phosphorylated on tyrosine. *B*, peak fractions (numbers 3 and 4) from *A* containing lipid rafts were mixed and run on SDS-PAGE, then immunoblots with the indicated antibodies were performed. Observations are representative of three experiments. *C*, Csk was immunoprecipitated from the lipid raft (*R*) and soluble (*S*) fractions after solubilization with 60 mM *n*-octyl-β-D-glucoside, precipitates were subjected to SDS-PAGE separation and anti-phosphotyrosine (*upper panel*), and anti-Csk (*lower panel*) immunoblots were performed. *D*, pervanadate-treated human T cells (5×10^8) were lysed in the presence of 60 mM *n*-octyl-β-D-glucoside to allow solubilization of lipid rafts, and the lysate was subjected to affinity chromatography with purified GST-Csk-SH3-SH2 fusion protein bound to glutathione-agarose beads; the column was washed extensively and eluted with 50 mM phosphotyrosine. Anti-phosphotyrosine and anti-PAG blots of the crude lysate and the phosphotyrosine eluate are shown and together with the data in *B* and *C* establish the identity of eluted protein as PAG/Cbp. Note the difference in mobility of the eluted band *versus* the band in lysate in the anti-PAG blot, since only tyrosine phosphorylated PAG/Cbp with lower mobility elutes from the column.

IL-2 Promoter Activity—To assess activity of the proximal IL-2 promoter, cells were transfected by electroporation with a construct consisting of the NFAT-AP-1 elements from the proximal IL-2 promoter inserted in a luciferase reporter construct, incubated with 5 μg/ml OKT-3 or 25 ng/ml PMA and 5 μM ionomycin for 6 h, lysed, and assayed for luciferase activity (Promega, Madison, WI).

RESULTS

Kinetics of Csk Dissociation from Lipid Rafts following T Cell Activation—The presence of Csk in rafts, together with the observation that Csk association with these membrane microdomains is regulated upon T cell activation via dephosphorylation of PAG/Cbp (22), suggest that Csk maintains a certain level of tonic inhibition of Src kinases, which is released during perturbation of the TCR. To address in more detail the level of inhibition by Csk, we first examined the kinetics of Csk dissociation and re-association with rafts. Purified T cells were stimulated by cross-ligation of the TCR/CD3, and samples were taken out at different time points, lysed, and subjected to lipid raft purification. In lipid rafts prepared from resting T cells (0

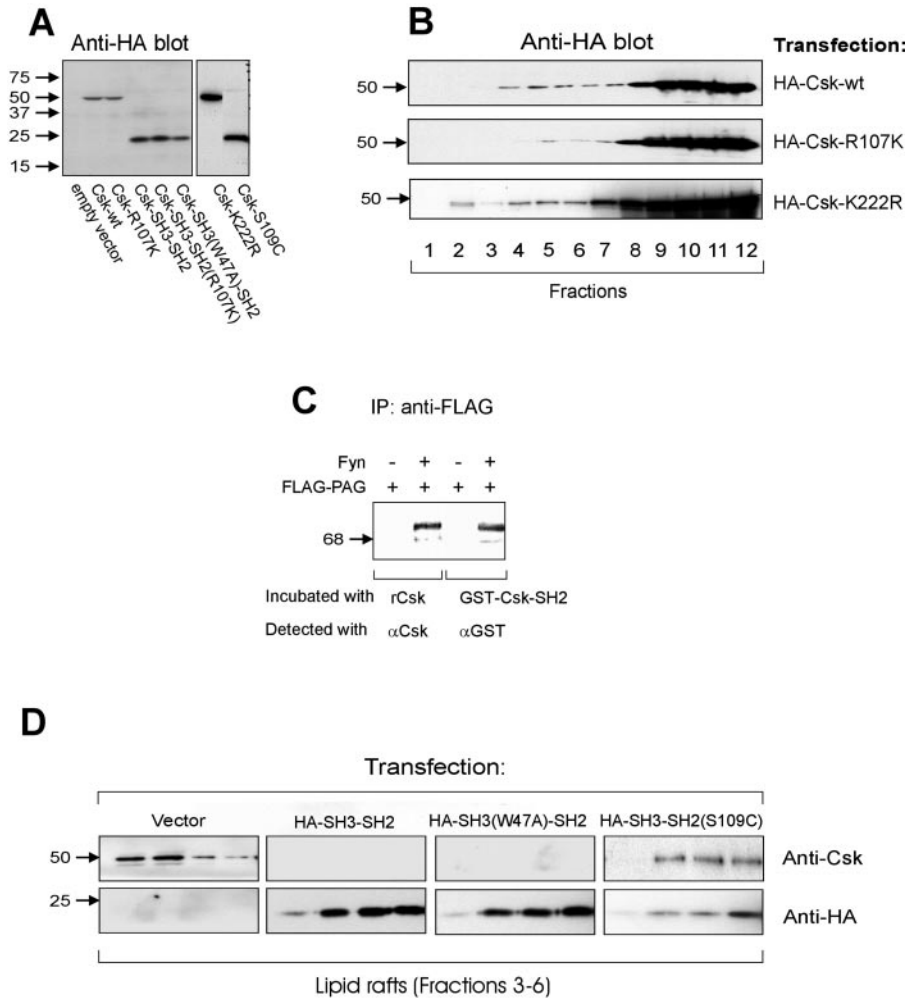

FIG. 2. **Overexpression of the Csk-SH2 domain displaces endogenous Csk from lipid rafts.** *A*, expression (anti-HA blot) of the different HA-tagged Csk constructs in transfected Jurkat TAg cells. *B*, anti-HA blot of fractions from lipid raft separation of Jurkat T cells transfected with HA-Csk, HA-Csk-K222R, which is a catalytically deficient mutant, or HA-Csk-R107K with a defective SH2 domain. *C*, FLAG-tagged PAG/Cbp was transfected into COS cells alone or together with Fyn as indicated, and anti-FLAG immunoprecipitations were performed. Subsequently, blots were overlaid with an Nonidet P-40 lysate of COS cells transfected with wild type Csk (*left*) or with recombinant Csk SH2 domain (*right*) and thereafter detected by anti-Csk. *D*, Jurkat T cells transfected with vector, HA-Csk-SH3-SH2, HA-Csk-SH3-SH2(S109C), or HA-Csk-SH3(W47A)-SH2 were subjected to lipid raft separation and the positions of HA-tagged, truncated, and endogenous Csk were assessed by anti-HA and anti-Csk immunoblots of the fractions.

min), we observed tyrosine-phosphorylated PAG/Cbp and Lck (Fig. 1*A*) as well as Csk (Fig. 1*B*). The identity of PAG/Cbp was established based on mobility, localization, immunoreactivity, and Csk SH2-association (Fig. 1, *B–D*). Upon T cell activation, we observed dephosphorylation of PAG/Cbp and phosphorylation of LAT at 2 min, whereas tyrosine-phosphorylated PAG/Cbp reappeared at 5 min (Fig. 1*A*). The kinetics of Csk association was assessed by analysis of fractions containing lipid rafts (numbers 3 and 4 pooled) at the different time points. Fig. 1*B* shows that whereas the amounts of PAG/Cbp remained constant, the majority of Csk was dissociated at 2 min following cross-ligation of the TCR/CD3 at the same time as dephosphorylation of PAG/Cbp occurred. However, concurrently with PAG/Cbp rephosphorylation, Csk rapidly reassociated with lipid rafts and was present with increasing levels at 5 and 10 min following activation. As expected, PLCγ_1 was recruited to rafts upon activation, whereas the amount of LAT in rafts did not appear to be regulated. Apparent minor differences in immunoreactive LAT levels at 2 min can be explained by the reduced ability of the anti-LAT antibody to recognize tyrosine-phosphorylated LAT (confer phospho-LAT blot in Fig. 1*B*), as previously observed with this antibody (33).

Overexpression of the Csk SH2 Domain Displaces Endogenous Csk from Lipid Rafts—Expression of Csk mutants lacking the kinase domain or with a kinase-dead mutation (K222R) (30), and with functional or deficient SH2 (wt, R107K or S109C (30, 31)) and SH3 domains (wt, W47A (32)) (Fig. 2*A*) show that a minor, but clearly detectable, fraction of Csk proteins with an intact SH2 domain (either full-length Csk-wt, Csk-K222R, or truncated Csk-SH3-SH2) are targeted to lipid rafts (Fig. 2, *B* and *D*). In contrast, mutation of critical residues for SH2 function (Arg^{107}, Ser^{109}) reduces raft localization. Furthermore, in far-Western analysis, PAG expressed in COS cells only interacted with wild type Csk or recombinant Csk SH2 domain when cotransfected with Fyn, indicating that the Csk SH2 domain requires a phosphotyrosine in PAG/Cbp for interaction (Fig. 2*C*, see also Fig. 1*D*). These observations support the notion that targeting of Csk to rafts is mediated only via interaction of the Csk SH2 domain with phospho-PAG/Cbp. Therefore, we next expressed the truncated Csk-SH3-SH2 or Csk-SH3(W47A)-SH2 at higher levels, which showed that these mutant proteins could compete out endogenous Csk from the lipid rafts (Fig. 2*D*). Presumably, displacement of wild type Csk occurs by saturating the SH2-binding phospho-Tyr^{317} in human PAG/Cbp, since the majority of the mutant Csk was in the soluble fractions numbers 7–12 (not shown). The soluble pool of wild type Csk (fractions numbers 7–12, data not shown) was only slightly affected by overexpression of mutant Csk, since the displaced, raft-associated pool is less than 5% of the total Csk (Ref. 34 and Fig. 2*B*).

Displacement of Csk from Lipid Rafts Leads to Constitutive and Sustained T Cell Activation—To assess the significance of the lipid raft-associated pool of Csk, we next examined the effect of overexpression of the truncated Csk (Csk-SH3(W47A)-SH2) with intact SH2 domain on proximal T cell activation in Jurkat T cells (Fig. 3*A*). Whereas activation of vector-transfected cells by anti-CD3 antibody leads to a rapid and transient tyrosine phosphorylation of the TCR ζ-chain and LAT, the level

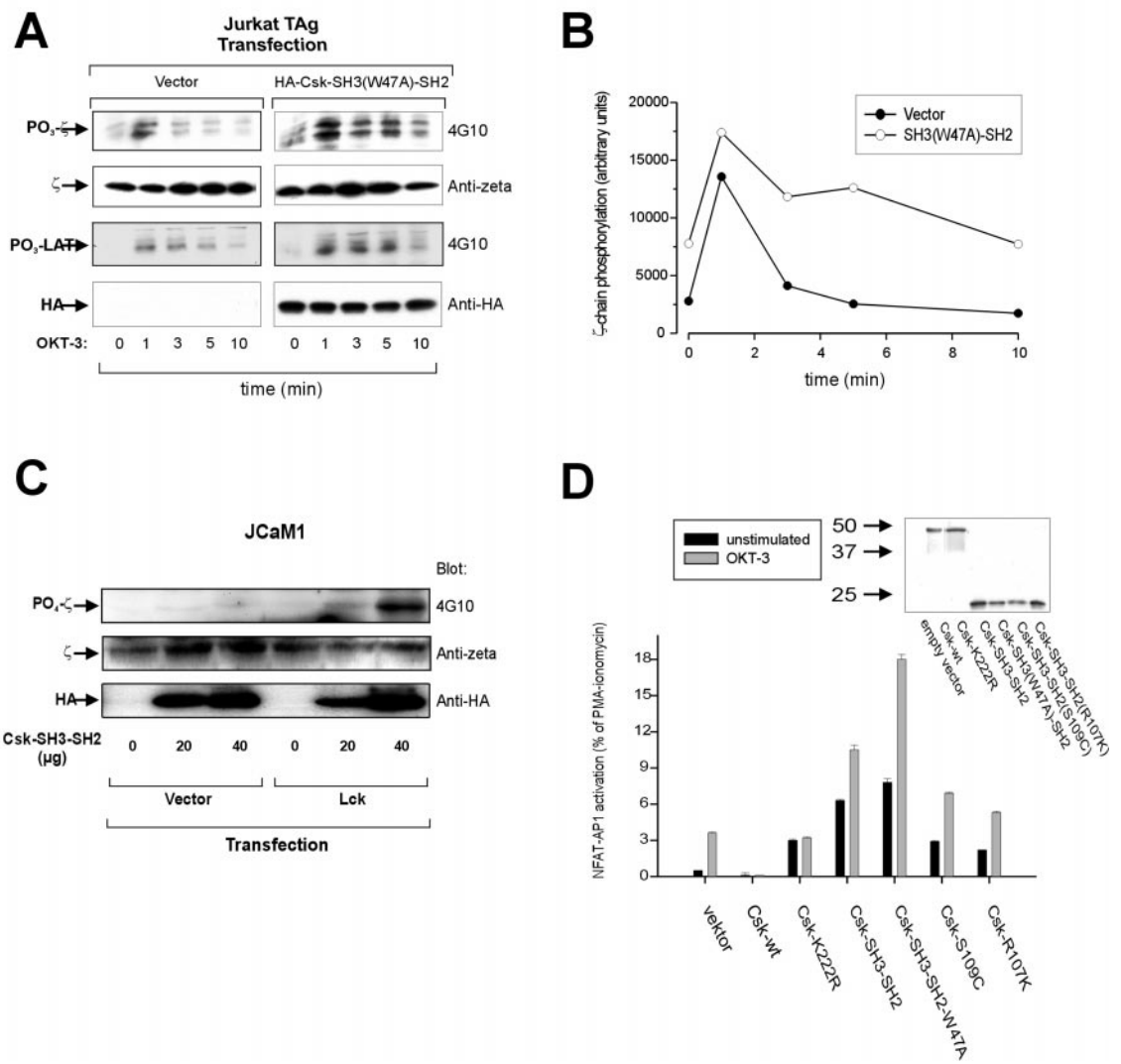

FIG. 3. **Overexpression of kinase-deficient Csk mutants is associated with elevated basal levels of T cell activation markers and sustained activity following TCR stimulation.** *A*, Jurkat TAg cells (2×10^7) were transfected with 60 μg of plasmid (either empty vector or encoding Csk-SH3(W47A)-SH2), harvested 20 h later, and washed twice. After 5 min of pre-equilibration at 37 °C, cells were stimulated with anti-CD3 antibodies (OKT-3) for the indicated time periods, lysed, and subjected to SDS-PAGE and immunoblotting with the indicated antibodies. *B*, densitometric scanning of tyrosine-phosphorylated ζ-chain shown in *A* as a function of time is presented for cells transfected with either empty vector (*filled circles*) or vector encoding Csk-SH3(W47A)-SH2 (*open circles*). Observations are representative of two experiments. *C,* Lck-defective JCaM1 Jurkat cells were transfected with increasing amounts of plasmid encoding Csk-SH3-SH2 (0–40 μg), together with empty vector or vector encoding wild type Lck, and analyzed as in *A*. *D,* overexpression of Csk mutants displacing endogenous Csk from lipid rafts is associated with increased NFAT-AP1-luciferase activity. Jurkat TAg cells (2×10^7) were cotransfected with NFAT-AP1-luciferase reporter construct (10 μg) and plasmid encoding the indicated Csk construct (60 μg), harvested 20 h later, and then incubated for 6 h untreated, treated with OKT-3, or treated with PMA/ionomycin. After lysis (and freezing) luciferase activity was measured using a luminometer. The level of NFAT-AP1 activation in cells transfected with the different constructs is presented as percent luciferase activity of that obtained by treatment with PMA and ionomycin of the same cells. *Inset*, expression of the transfected constructs. One representative of three or more experiments is presented.

of phosphorylation of both molecules was higher and more sustained in cells overexpressing mutant Csk. Furthermore, basal levels of ζ-chain phosphorylation were elevated in resting Csk-SH3(W47A)-SH2-expressing cells (Fig. 3, *A* and *B*). Similar effects on TCR-induced ζ-chain phosphorylation were observed with other Csk mutants (Csk-K222R and Csk-SH3-SH2) being able to displace endogenous Csk from lipid rafts (data not shown). To address the basal level of ζ-chain phosphorylation in more detail without background from nontransfected cells, JCaM1 Jurkat cells with defective Lck were transfected with increasing amounts of Csk-SH3-SH2 together with wild type Lck or empty vector. Phosphorylation of the ζ-chain could then be induced by anti-CD3 antibody in the Lck-transfected cells (not shown). In resting cells, ζ-chain phosphorylation was absent in cells transfected with Lck alone ((Fig. 3*C, fourth lane*) but strongly induced by expression of increasing amounts of Csk-SH3-SH2 (*fifth* and sixth *lanes*), whereas no such effect was seen in the absence of Lck (*first*, *second*, and *third lanes*).

To assess downstream T cell activation events with relevance to T cell function, Jurkat T cells were transfected with a luciferase reporter directed by the proximal IL-2 promoter containing the NFAT and AP1 response elements together with constructs directing expression of various Csk mutants (Fig. 3*D*). Cells transfected with empty vector displayed low basal levels of NFAT-AP1 reporter activity and a TCR-induced increase in activity, while overexpression of wild-type Csk, as expected, completely abolished reporter activity. In contrast, cells transfected with constructs directing expression of Csk mutants with an intact SH2 domain (Csk-K222R, Csk-SH3-SH2, or Csk-SH3(W47A)-SH2), and with the capacity to displace endogenous Csk from lipid rafts, exhibited severalfold elevated levels of NFAT-AP1 reporter activity in the absence

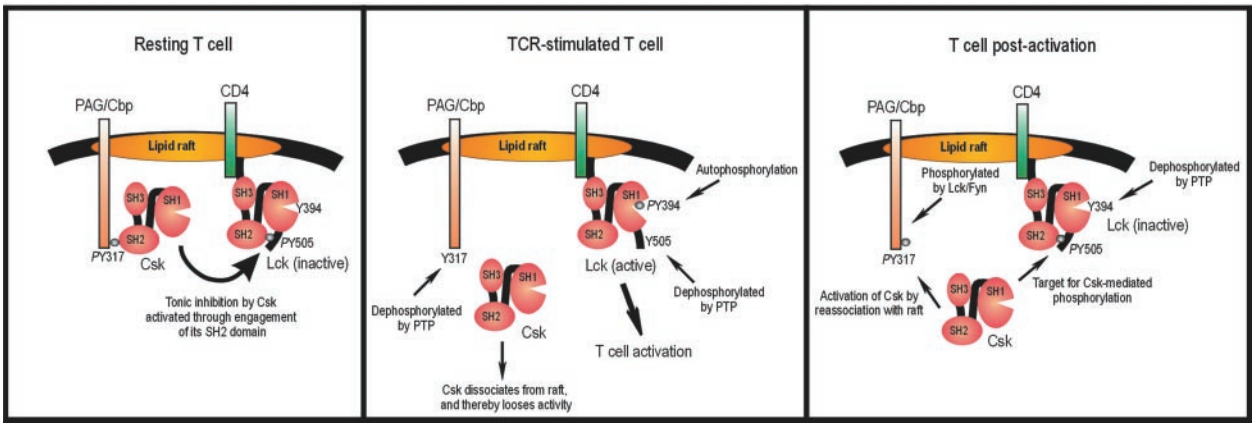

FIG. 4. **Csk is temporarily sent off duty during T cell activation.** In resting normal T cells (*left panel*), Csk is present in lipid rafts through interaction with Tyr^{317} on PAG/Cbp. This imposes a tonic inhibition of T cell activation through Csk-mediated phosphorylation of the Lck regulatory site (Tyr^{505}). Engagement of the T cell receptor (*middle panel*) leads to dephosphorylation of PAG/Cbp by an unknown PTPase, dissociation of Csk from lipid rafts, and displacement from its substrate Lck, leading to activation of Lck and initiation of the TCR-induced tyrosine phosphorylation cascade. However, after 2–5 min of activation (*right panel*), PAG/Cbp Tyr^{317} is re-phosphorylated by Lck and/or Fyn, thereby recruiting Csk back into lipid rafts. This terminates Lck and Fyn activity and turns off TCR signaling. Reproduced from Ref. 38 with permission from Cellular Signaling.

of TCR stimulation. In cells transfected with Csk-SH3-SH2 or Csk-SH3(W47A)-SH2, stimulation through TCR activated the NFAT-AP1 reporter to higher total levels, although the elevated basal levels reduced the relative effect of TCR stimulation. Surprisingly, cells transfected with Csk-K222R in three independent experiments displayed no induction in reporter activity upon TCR stimulation, although basal levels were substantially increased. It may be speculated that mutated full-length Csk has a better conformation of the SH3 domain, which interacts more strongly with other proteins (*e.g.* PEP). In contrast, the Csk-SH3(W47A)-SH2 that does not recruit tyrosine phosphatases gave strong TCR-mediated activation (W47A > SH3-SH2 > K222R). Alternatively, the increased basal level but lack of further stimulation through TCR observed with the Csk-K222R mutation in the ATP binding site may be due to substrate trapping (*i.e.* Lck is sequestered) or due to other Csk kinase domain interaction partners. Transfection of constructs with mutated SH2 domains (Csk-SH3-SH2(R107K) and Csk-SH3-SH2(S109C)) gave NFAT-AP1 reporter activities that were significantly lower than those of the Csk-SH3-SH2 construct but higher than basal levels. This is probably due to the presence of residual phosphotyrosine binding capacity related to these mutants (Fig. 2*D* and Refs. 30 and 31).

DISCUSSION

Unlike Src family PTKs, Csk does not have a C-terminal negative regulatory tyrosine phosphorylation site and therefore cannot be suppressed by the intramolecular "tail-bite" mechanism that is characteristic for Src-like kinases (35). Indeed, it appears that Csk is constitutively active and exerts a tonic suppression of the Src family kinases Lck and Fyn in the lipid rafts of resting T cells (Fig. 4, *left panel*). Such basal levels of inhibition appear to set the threshold for T cell activation to prevent an aberrant immune response (20). In the presence of this tonic negative regulation, T cell activation requires two independent early steps: 1) initiation of activating pathways and 2) release from inhibition. A molecular mechanism for the latter is apparently provided by the TCR-induced, specific, and rapid dephosphorylation of PAG/Cbp, resulting in dissociation of Csk from the lipid rafts where Lck and Fyn reside (22) (Fig. 4, *middle panel*). This allows a stronger and prolonged activation of Lck and Fyn and an improved TCR signaling. However, the relief is only temporary in that PAG/Cbp is again phosphorylated after several minutes and recruits Csk back to the lipid raft environment, where it can suppress the Src family PTKs (Fig. 4, *right panel*). If PAG/Cbp is phosphorylated by Lck and/or Fyn as proposed (22), this mechanism is a classical negative feedback loop that would limit the time course of tyrosine phosphorylation of substrates for Lck and Fyn.

Our observations (Ref. 22 and this paper) imply that a protein-tyrosine phosphatase (PTPase) responds to TCR ligation by dephosphorylating PAG/Cbp. This so far unidentified enzyme may also be present in lipid rafts. Dephosphorylation apparently occurs at all major physiological tyrosine phosphorylation sites as shown by anti-phosphotyrosine immunoblotting. One candidate for the PTPase that removes phosphate from PAG/Cbp is CD45, which also directly activates Lck and Fyn (36). However, unpublished data[2] show that PAG/Cbp dephosphorylation also occurs in the CD45-deficient J45.01 Jurkat T cells (22). The phosphatase PEP, which can be complexed with Csk through binding to its SH3 domain (37), is another obvious candidate. PEP may also contribute to tonic inhibition of Lck and Fyn by dephosphorylating the positive regulatory phosphotyrosines in Lck (Tyr^{394}) (7) and Fyn-T (Tyr^{416}) (8). Release of Csk from rafts would in addition remove the associated PEP and thereby improve the phosphorylation of Lck and Fyn at their activating sites. Overexpression of Csk-SH3(W47A)-SH2, which should not bind PEP due to mutation of the SH3 domain, affected NFAT-AP-1 activation more strongly than expression of Csk-SH3-SH2, suggesting a role for PEP or other interaction partners in lipid rafts. Nevertheless, the constitutive promoter activation in cells expressing either of these mutants indicates the important role of Csk in repression of the Src kinases in resting T cells.

In conclusion, we show that Csk localized to lipid rafts via its SH2 domain is constitutively inhibiting T cell activation. When T cells are activated through the TCR, Csk rapidly dissociates from lipid rafts leading to release from inhibition. Shortly thereafter, Csk reassociates, which contributes to shutting down the TCR signaling. Csk thus functions as a regulatory gatekeeper for T cell activation.

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