Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor

By Torkel Vang,* Knut Martin Torgersen,* Vibeke Sundvold,‡ Manju Saxena,§ Finn Olav Levy,* Bjørn S. Skålhegg,* Vidar Hansson,* Tomas Mustelin,§‖ and Kjetil Taskén*

*From the *Department of Medical Biochemistry, Institute of Basic Medical Sciences, University of Oslo, N-0317 Oslo, Norway; the ‡Institute of Immunology, University of Oslo, The National Hospital, N-0027 Oslo, Norway; the §La Jolla Institute for Allergy and Immunology, San Diego, California 92121; and the ‖La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037*

Abstract

In T cells, cAMP-dependent protein kinase (PKA) type I colocalizes with the T cell receptor–CD3 complex (TCR/CD3) and inhibits T cell function via a previously unknown proximal target. Here we examine the mechanism for this PKA-mediated immunomodulation. cAMP treatment of Jurkat and normal T cells reduces Lck-mediated tyrosine phosphorylation of the TCR/CD3 ζ chain after T cell activation, and decreases Lck activity. Phosphorylation of residue Y505 in Lck by COOH-terminal Src kinase (Csk), which negatively regulates Lck, is essential for the inhibitory effect of cAMP on ζ chain phosphorylation. PKA phosphorylates Csk at S364 in vitro and in vivo leading to a two- to fourfold increase in Csk activity that is necessary for cAMP-mediated inhibition of TCR-induced interleukin 2 secretion. Both PKA type I and Csk are targeted to lipid rafts where proximal T cell activation occurs, and phosphorylation of raft-associated Lck by Csk is increased in cells treated with forskolin. We propose a mechanism whereby PKA through activation of Csk intersects signaling by Src kinases and inhibits T cell activation.

Key words: protein kinase A • Csk • T cell activation • tyrosine phosphorylation • immunomodulation

Introduction

Engagement of the TCR/CD3 complex leads to activation of the Src family tyrosine kinases Lck and Fyn (1, 2). These kinases mediate the initial tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs in the TCR/CD3 subunits (e.g., ζ chain) and elicit a complex series of proximal signaling events. This involves recruitment of the tyrosine kinase Zap-70 to the ζ chain and subsequent tyrosine phosphorylation of lipid raft–associated adaptor molecules such as the linker for activation of T cells (LAT)[1] that, via phosphotyrosine binding, further recruit several downstream, Src homology 2 (SH2) domain–containing signaling molecules (for a review, see reference 3). The Src family of tyrosine kinases are negatively regulated by phosphorylation of a conserved COOH-terminal tyrosine residue (Y505 in Lck, Y528 in FynT) by the COOH-terminal Src kinase, Csk (4–6). Although Csk has substantial homology to Src kinases, it lacks the COOH-terminal regulatory tyrosine found in Src kinases (7). Little or no evidence has been presented to demonstrate any enzymatic regulation of Csk (8), such as by other signaling pathways. However, a

K.M. Torgersen and V. Sundvold contributed equally to this work.

F.O. Levy's present address is Merck, Sharp, and Dohme Cardiovascular Research Center and Institute of Pharmacology, University of Oslo, Rikhospitalet University Hospital, N-0316 Oslo, Norway. B.S. Skålhegg's present address is Dept. of Nutrition Research, University of Oslo, N-0317 Oslo, Norway.

Address correspondence to Tomas Mustelin, La Jolla Cancer Center, The Burnham Institute, 70901 North Torrey Pines Rd., La Jolla, CA 92037. Phone: 858-713-6270; Fax: 858-713-6274; E-mail: tmustelin@burnham-inst.org

[1]*Abbreviations used in this paper:* Cbp, Csk binding protein; Csk, COOH-terminal Src kinase; HA, hemagglutinin; IBMX, isobutyl-methylxanthine; LAT, linker for activation of T cell; PAG, phosphoprotein associated with glycosphingolipid-enriched membrane domains; PKA, protein kinase A or cAMP-dependent protein kinase; PKI, protein kinase inhibitor; SH2, Src homology 2.

recently identified LAT-homologous, transmembrane adaptor protein called Csk-binding protein (Cbp; reference 9) or phosphoprotein associated with glycosphingolipid-enriched membrane domains (PAG; reference 10) with the capacity to bind Csk, may allow spatial regulation of Csk activity toward Src kinases in lipid rafts (8), and binding of Csk to Y317 in Cbp/PAG may increase the activity of Csk (11).

cAMP, the levels of which are increased, for example, by prostaglandin E and β-adrenergic stimuli, negatively regulates mitogenic signaling pathways at multiple levels (12–14). In normal T cells, cAMP-dependent protein kinase (PKA) type I colocalizes with the TCR–CD3 complex and inhibits TCR-induced cell proliferation via a previously unknown proximal target (15–17). In T cells from HIV-infected patients and some patients with common variable immunodeficiency, increased levels of cAMP and hyperactivation of PKA type I contribute to the T cell dysfunction, and PKA type I selective antagonists can improve immune function of patient T cells in vitro up to 300% (18–20). Now, we report a novel inhibitory pathway in T cells whereby PKA type I, through activation of Csk leading to inhibition of Lck-mediated ζ chain phosphorylation, shuts down the proximal T cell activation. Furthermore, we demonstrate that the whole PKA type I-Csk-Lck inhibitory pathway spatially is assembled in lipid rafts where the initial T cell activation takes place.

Materials and Methods

Cell Culture, Stimulation, and Transfection. The human leukemic T cell line Jurkat (clone E6.1), Jurkat TAg, a derivate of the Jurkat cell line stably transfected with the SV40 large T antigen (21), and the Lck-deficient JCaM1 cell line (22) were kept in logarithmic growth in RPMI 1640 supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, and monothioglycerol. Human peripheral blood T cells were purified from normal donors by negative selection (18). T cells were activated by the addition of 5–10 μg/ml of anti-CD3ε mAb OKT-3 or by pervanadate treatment. For transfections, cells (2×10^7) in 0.4 ml Opti-MEM were mixed with 2–80 μg of each DNA construct in electroporation cuvettes with a 0.4-cm electrode gap (BioRad Laboratories) and subjected to an electric field of 250 V/cm with 960 μF capacitance. The cells were expanded in complete medium and harvested after 20 h. To obtain only the transfected cell population for functional assays, cells were cotransfected with a plasmid encoding the rat NK cell marker NKR-P1A (a gift from Dr. J.C. Ryan, VA Medical Center, University of California at San Francisco, San Francisco, CA) and purified by positive selection using anti–rat NKR-P1 mAb (clone 3.2.3) and anti–mouse IgG paramagnetic beads which allows release of bead-bound cells by digestion of a DNA linker that attaches the Ab to the bead (Cellection; Dynal).

Immunoprecipitations. Immunoprecipitation of Zap-70, Lck, and Csk was as described previously (23). For immunoprecipitation of hemagglutinin epitope (HA)-tagged Csk, transfected cells were disrupted in lysis buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, and 10 μg/ml each of leupeptin, antipain, pepstatin A, and chymostatin). When cells were stimulated with OKT-3, cell lysates were precleared by incubation with protein A/G–Sepharose beads (Sigma-Aldrich) for 1 h at 4°C, and subjected to immunoprecipitation with anti-HA mAb (Babco) or anti-Csk Ab (Santa Cruz Biotechnology, Inc.). 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 three times in Csk kinase assay buffer (50 mM Hepes, 5 mM $MgCl_2$, pH 7.4), followed by Csk kinase assays and Western blot analysis.

Immunoblot Analysis. Detection of phosphotyrosine by anti-PTyr mAb (4G10; Upstate Biotechnology), and immunoblotting with anti–Zap-70, anti-Lck, anti-HA, anti-Csk, anti-PKA RIα, anti-PKA RIIα, anti-PKA C, and anti-LAT Abs were as before (18, 23, 24) except that recently developed mAbs directed against human RIα and human RIIα (cat. no. P53620; K. Taskén in collaboration with Transduction Laboratories) and anti-Csk Ab from Santa Cruz Biotechnology, Inc. (SC-286) were used.

Plasmid Constructs. The gene-encoding human Csk (25) was subcloned into the expression vector pEF-BOS/HA at NheI-XbaI sites. Csk-S364A, Csk-S364C, and Csk-S339A/S340A/T341A mutants were made by PCR or using a site-directed mutagenesis kit (Quickchange; Stratagene) and verified by sequencing.

Expression of Recombinant Enzymes. Cloning, expression, and purification of human Csk has been reported previously (25) and yielded an enzyme with a specific activity in the range of that of the native purified enzyme. Recombinant purified catalytic subunit of PKA (Cα; reference 26) was a gift from Dr. F. Herberg, Ruhr University, Bochum, Germany.

Phosphorylation of Csk. Csk was incubated with PKA C subunit at 30°C for the indicated time periods in 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 3–5 μM [γ-^{32}P]ATP (50–320 Ci/mmol). All reactions were stopped by boiling samples in SDS sample buffer, followed by SDS-PAGE. Gels were stained with Coomassie brilliant blue, dried, and subjected to autoradiography.

In Vitro Tyrosine Kinase Assays. The tyrosine kinase activity of human Csk was measured as incorporation of [^{32}P]phosphate into the synthetic polyamino acid poly(Glu,Tyr) 4:1 (Sigma-Aldrich), abbreviated pEY. A standard protocol was followed (25) with reaction volumes of 50 μl containing Hepes buffer, pH 7.4, 5 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (0.15 Ci/mmol), 200 μg/ml pEY, and different amounts of purified Csk. Native or heat-inactivated (65°C for 10 min) C subunit and/or protein kinase inhibitor peptide (protein kinase inhibitor [PKI] 6-22 amide; Sigma-Aldrich) was added where indicated. The incubation temperature was 30°C, and the incubation times were 12–15 min, if not otherwise stated.

Phosphoamino Acid Analysis. Csk was phosphorylated by PKA for 30 min as indicated above and subjected to SDS-PAGE. The band corresponding to phosphorylated Csk was cut from the dried gel and subjected to partial acid hydrolysis in 6 M HCl at 110°C for 2 h. The acid was evaporated under vacuum and the hydrolyzed sample was dissolved in 30 μl H_2O. 10 μl of sample (~1,000 cpm of ^{32}P) was separated in two dimensions together with 10 μg each PSer, PThr, and PTyr. Phosphoamino acid standards were stained with ninhydrin, and ^{32}P-labeled amino acids were detected by autoradiography.

IL-2 Production Assay. Cell-free supernatants were harvested from Jurkat T cells after 20 h of culture and stored at −80°C. IL-2 levels were determined by ELISA (R&D Systems).

Lipid Raft Purification. Isolation of lipid rafts or glycolipid-enriched membrane microdomains was performed as described in detail elsewhere (27). In brief, cells were homogenized in 1 ml ice-cold lysis buffer (described above) by 10 pestle strokes in a

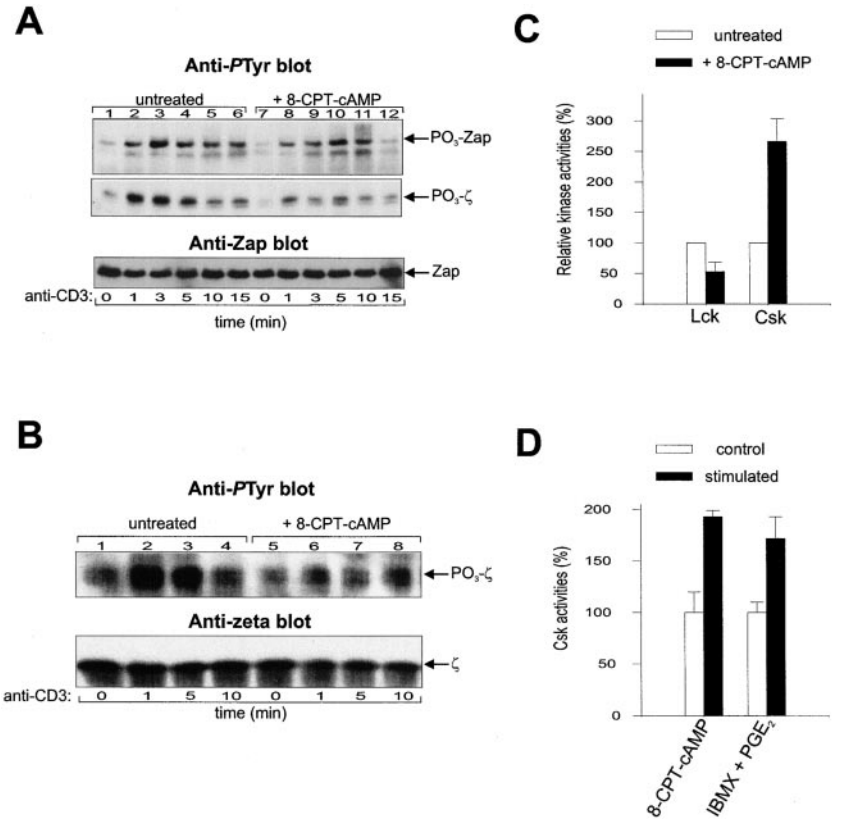

Figure 1. cAMP inhibits TCR/CD3-induced ζ chain phosphorylation. (A) The phosphotyrosine content of Zap-70 and ζ chain were examined (top and middle, respectively) in anti–Zap-70 immunoprecipitates from untreated (lanes 1–6) and 8-CPT-cAMP–pretreated (300 μM for 30 min; lanes 7–12) Jurkat cells stimulated with anti-CD3 Ab (OKT-3) for the indicated time (0–15 min). Anti–Zap-70 immunoblotting verified equal amounts of Zap-70 (bottom). (B) ζ chain phosphorylation (top) was examined in peripheral T cell lysates after anti-CD3 Ab stimulation (0–10 min) of untreated (lanes 1–4) and 8-CPT-cAMP–pretreated (500 μM for 15 min; lanes 5–8) cells. Anti–ζ chain immunoblotting verified equal loading (bottom). (C) Tyrosine kinase activities of Lck and Csk were assessed in immunoprecipitates of Jurkat T cells either treated with 8-CPT-cAMP (300 μM) for 20 min (black bars) or untreated (white bars; means ± SEM). Immunoblotting verified comparable amounts of Lck and Csk in immunoprecipitates from 8-CPT-cAMP–treated and untreated cells. (D) Csk activities in untreated peripheral T cells (white bars) and cells treated with 8-CPT-cAMP (500 μM for 5 min) or IBMX (200 μg/ml for 15 min) together with PGE2 (100 μM for 5 min; black bars) were assessed as in C. In parallel experiments, PGE2 treatment produced cAMP levels that increased from 1.6 ± 0.4 to 20.8 ± 7.3 pmol/10^6 cells. For the data presented in A and B, one representative of three experiments is shown.

Dounce homogenizer, loaded at the bottom of a 40–5% sucrose gradient and centrifuged at 200,000 *g* for 20 h. 0.4-ml fractions were collected from the top.

Results

cAMP Inhibition of ζ Chain Phosphorylation Is Dependent on the COOH-terminal Regulatory Tyrosine 505 in Lck. cAMP treatment of Jurkat T cells (Fig. 1 A) and normal peripheral blood T cells (Fig. 1 B) inhibited and delayed the tyrosine phosphorylation of TCR-ζ chain and Zap-70 after T cell activation by anti-CD3 (OKT3; compare lanes 2 and 8 in Fig. 1 A and lanes 2 and 6 in Fig. 1 B). ζ chain and Zap-70 represent good in vivo substrates for Lck, and their phosphorylation status can be readily assessed in detergent-solubilized extracts as the ζ chain is only loosely associated with lipid rafts in activated T cells (28). Examination of Lck immune precipitates from cAMP-treated Jurkat T cells also showed a 50% decrease in kinase activity in vitro (Fig. 1 C). However, no direct downregulation of Lck or Fyn activity by PKA could be observed in immune precipitates or on purified Lck (data not shown). In contrast, Csk activity was increased two- to threefold after cAMP treatment. Furthermore, cAMP or PGE2 in combination with isobutyl-methylxanthine (IBMX) increased Csk activity similarly in peripheral T cells (Fig. 1 D). Transfection of JCaM1 cells that have a truncated and inactive Lck (Fig. 2, lanes 1–4) with wild-type Lck (lanes 5–8) or Lck-Y505F (lanes 9–12) reconstituted TCR-mediated signaling as evident from anti-CD3–induced ζ chain phosphorylation. Whereas cells with wild-type Lck showed a distinct reduction in anti-CD3–induced phosphorylation of ζ chain when pretreated with 8-CPT-cAMP (top panel, compare lane 8 with lane 6), ζ chain phosphorylation was not inhibited by cAMP in cells with Lck-Y505F (compare lane 12 with lane 10). We conclude that the regulatory site Y505 of Lck is required for cAMP-mediated inhibition of ζ chain phosphorylation.

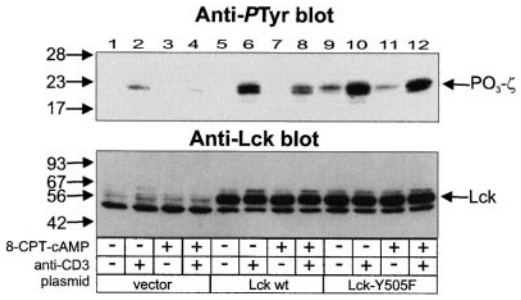

Figure 2. cAMP-mediated inhibition of TCR/CD3-induced ζ chain phosphorylation is dependent on inactivation of Lck by phosphorylation at Y505. Lck-deficient JCaM1 cells were transfected with empty pEF vector alone (vector), with wild-type (wt) Lck, or with mutant Lck-Y505F, where the COOH-terminal Y505-regulatory site is mutated to resist inactivation by Csk, and incubated (30 min) in the absence or presence of 8-CPT-cAMP (300 μM) followed by incubation in the absence or presence of anti-CD3 Ab (5 min). PO_3-ζ chains (top) were fished with GST-Zap-70-$(SH2)_2$ and detected by phosphotyrosine immunoblotting (reference 40). All lanes contain the endogenous truncated Lck present in JCaM1 cells (catalytically inactive; lower bands), whereas full-length Lck is present in equal amounts in transfected cells (lower panel; lanes 5–12). One representative of three experiments is shown.

This implicates Csk as a target for regulation by PKA, and we next explored that possibility.

PKA Phosphorylation Activates Csk. Fully active recombinant Csk (25) was readily phosphorylated by Cα of PKA (Fig. 3 A; lane 1, arrow), whereas no phosphorylation of Csk was detected when incubated with heat-inactivated (65°C for 10 min) Cα (lane 2). Incubation of recombinant Csk with the recombinant catalytic subunit of PKA (Cα) more than doubled the Csk-catalyzed phosphorylation of pEY compared with Csk incubated alone (Fig. 3 B, compare bar 2 with bar 1). This effect was not seen with heat-inactivated Cα (bar 3). Furthermore, the increase in Csk activity in the presence of native Cα was strongly reduced by the addition of PKI, a specific inhibitor of PKA (bar 4). PKA itself did not phosphorylate pEY (data not shown). In the presence of heat-inactivated Cα, Csk activity was constant for the first 10 min and then declined, whereas the activity curve was much steeper in the presence of native Cα and the activity was approximately twofold higher at each time point (Fig. 3 C). Increasing concentrations of Cα subunit led to a saturable increase in activation of Csk, reaching a maximum around a twofold molar excess of C subunit over Csk (Fig. 3 D). Incubation of pEY with increasing concentrations of Csk demonstrated a concentration-dependent increase in phosphate transfer, which was approximately twofold higher at all concentrations in the presence of a fixed amount of native C (Fig. 3 E).

To look at a normal substrate for Csk, heat-inactivated Lck was used as substrate and the activity of Csk in the presence and absence of PKA was examined. When Csk was limiting in the reaction, Csk-mediated tyrosine phosphorylation of Lck was 4.8-fold stronger in the presence than in the absence of PKA (Fig. 4).

Phosphorylation of Csk-S364 Is Necessary for the PKA Regulation of Csk in Intact T Cells. Phosphoamino acid analysis of Csk phosphorylated by PKA demonstrated strong labeling on phosphoserine (Fig. 5 A). Tryptic peptide mapping of Csk phosphorylated by PKA revealed two major radioactive spots both of which contained PSer (Fig. 5 B, peptides 1 and 2). The human Csk amino acid sequence con-

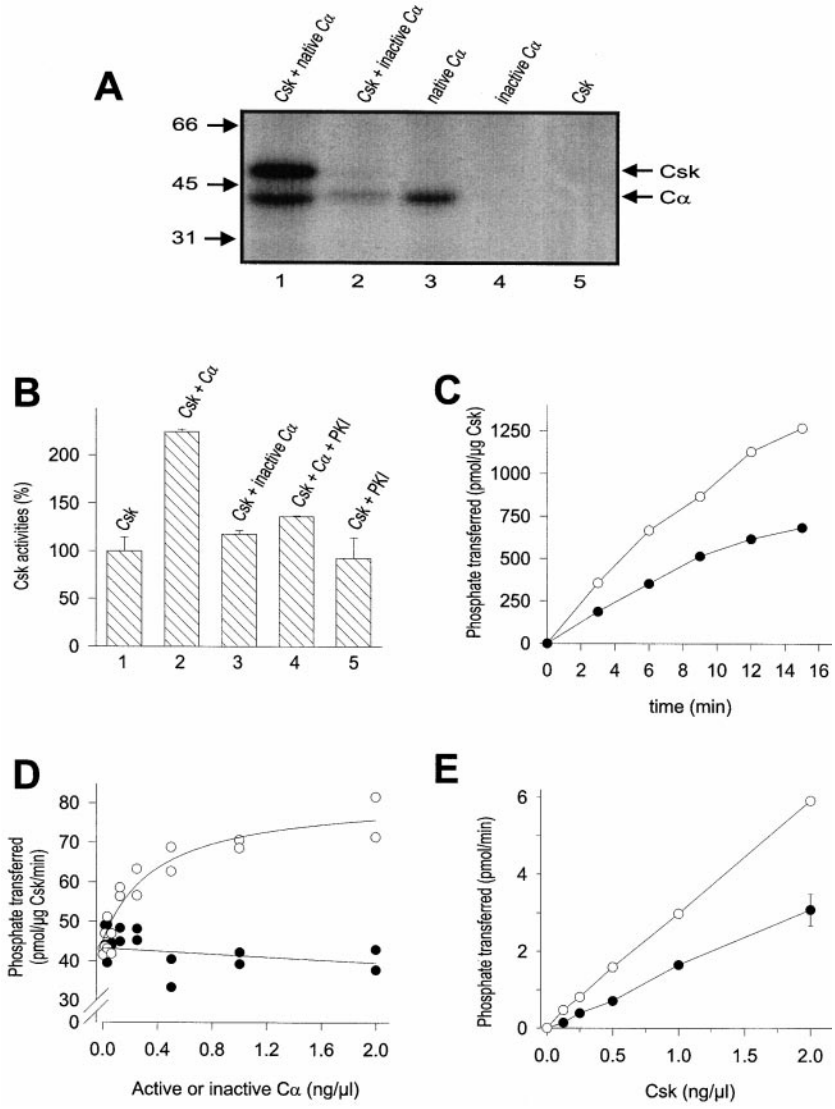

Figure 3. PKA-mediated phosphorylation increases the tyrosine kinase activity of Csk. (A) Csk (10 ng/μl) was incubated with native Cα (5 ng/μl active; lane 1) and heat-inactivated (65°C for 10 min) Cα (lane 2) and [γ-^{32}P]ATP and subjected to SDS-PAGE and autoradiography. Native Cα alone (lane 3), heat-inactivated Cα alone (lane 4), and Csk incubated alone (lane 5) were included as controls. Arrows indicate phosphorylated Csk (50 kD) and autophosphorylated Cα (40 kD). (B) Csk (1 ng/μl) kinase activity when incubated alone (1), in the presence of native (2), or heat-inactivated (65°C for 10 min) (3) Cα (2 ng/μl; means ± SD, $n = 5$). Coincubation of Csk and PKI (85 μM) with (4) or without (5) native Cα is also shown. (C) Time-dependent phosphorylation of pEY by Csk (1 ng/μl) in the presence of native (○) and heat-inactivated (65°C for 10 min) (●) Cα (2 ng/μl). Each experiment was performed with single point measurements, and one representative of a total of seven assays is shown. (D) The effects of different amounts (0–2 ng/μl) of native (○) and heat-inactivated (●) Cα on Csk (1 ng/μl)-catalyzed phosphate transfer to pEY. Duplicate measurements were performed, and one representative assay of a total of four is shown. (E) Csk (0–2 ng/μl) concentration-dependent phosphotransfer in the presence of a constant amount of native (2 ng/μl active; ○) or heat-inactivated (●) Cα. All samples were assayed in duplicate and error bars (half range) are shown. Where error bars are not visible, they are within the point. One representative experiment of four is presented.

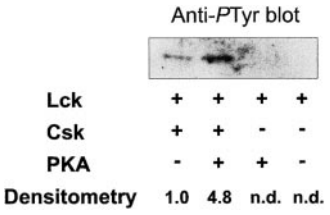

Figure 4. PKA phosphorylation increases the tyrosine kinase activity of Csk towards an endogenous substrate. Tyrosine phosphorylation of heat-inactivated (65°C for 10 min) purified Lck enzyme (30 ng/μl, cat. no. 14-106; Upstate Biotechnology) by Csk (0.3 ng/μl) was assessed either in the presence or absence of PKA catalytic subunit Cα (10 ng/μl) in a buffer containing 5 mM Mg^{2+} and 200 μM ATP at 30°C for 10 min. Reactions were stopped by the addition of SDS sample buffer, subjected to SDS-PAGE, and phosphotyrosine content of Lck was assessed by antiphosphotyrosine immunoblotting (4G10). Densitometric scanning was performed to evaluate the level of Csk-mediated tyrosine phosphorylation of Lck in the absence and presence of PKA. n.d., not done.

tains one putative phosphorylation site that fits the motif preferred by PKA, at amino acids 361–364 in the sequence KKFS. A Csk-S364A mutant was only weakly phosphorylated by PKA (Fig. 5 B, and data not shown), and both major tryptic peptides (1 and 2) were missing compared with wild-type Csk phosphorylated by PKA (Fig. 5 B). The observation that two phosphorylated peptides disappeared by mutation of a single residue is probably because of partial proteolysis by trypsin. To assess the phosphorylation of Csk in intact cells, Jurkat T cells were metabolically labeled with ^{32}Pi, and anti-Csk immunoprecipitates were analyzed by tryptic peptide mapping (Fig. 5 C). Whereas Csk from untreated cells contained a few weakly labeled phosphopeptides, treatment with cAMP or PGE1 induced the appearance of one strong (peptide 1) and three weaker spots (peptides 2–4). Peptides 1 and 2 comigrated with those in Fig. 5 B, as shown by eluting peptides from the maps with PGE1-induced in vivo–labeled and recombinant Csk and rerunning mixtures with equal amounts of radioactivity on new maps (Fig. 5 D). To determine the site of phosphorylation by PKA in intact cells, Jurkat cells transfected with HA-tagged wild-type and mutant Csk were metabolically labeled and then stimulated with cAMP (Fig. 5 C, right two panels). Tryptic peptide mapping revealed that whereas HA-Csk phosphopeptides (1, 3, and 4) comigrated with those of endogenous Csk, an S364C mutation abrogated labeling of peptide 1. The Csk-S364C mutant was catalytically active both when expressed in *Escherichia coli* and Jurkat TAg cells (Fig. 6, A and C), whereas Csk-S364A was not. Perhaps Cys, but not Ala, in position 364 permitted a normal folding of Csk. Another site at the activation loop of Csk in the sequence KEASST (amino acids 336–341) could also potentially be phosphorylated by PKA, although not fully consistent with the motif preferred by PKA. This last region is often the site of kinase activation by autophosphorylation (29) or transphosphorylation by another kinase, for example, mitogen-activated protein kinase (MAPK) activation by MAPK kinase (30). The extent of PKA-mediated phosphorylation of a Csk-S339A/S340A/T341A mutant (Csk-AAA) was comparable to that of wild-type Csk, and its tryptic peptide map was identical to that of wild-type (data not shown). A PKA-mediated increase in the kinase activity of this latter mutant and wild-type, but not

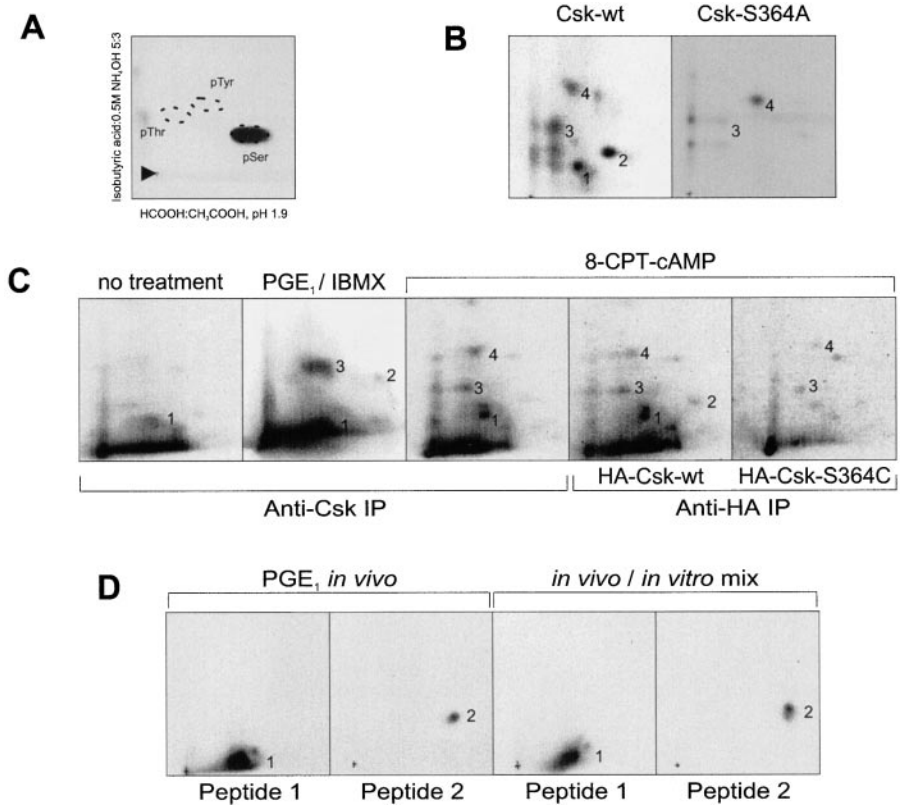

Figure 5. Mapping of Ser364 in Csk as a phosphorylation site for PKA in T cells. (A) Phosphoamino acid analysis of Csk phosphorylated by PKA. Csk (3 ng/μl) was incubated with PKA (GST-Cβ; 1.5 ng/μl active). (B) Wild-type (wt) Csk and mutant Csk-S364A were phosphorylated by Cα in vitro and subjected to tryptic peptide mapping (reference 41). (C) Tryptic peptide maps of ^{32}Pi-Csk obtained by metabolic labeling of Jurkat T cells (reference 42). Endogenous Csk was immunoprecipitated from untreated, 8-CPT-cAMP–treated (300 μM for 30 min), or PGE1/IBMX-treated (10 μM/200 μg/ml for 5 min) cells. HA-tagged Csk or Csk-S364C was immunoprecipitated with an anti-HA mAb from transfected cells treated with 8-CPT-cAMP (300 μM for 30 min). To allow for production of enough material after excision of spots 1 and 2 for the rerunning experiment presented in D, higher levels of radioactivity were used in the experiment conducted with PGE1/IBMX-treated cells. (D) Peptides 1 and 2 from the PGE1-treated cells were excised from the gel and run again either alone (left panels) or mixed with peptides 1 and 2 from the experiment in Fig. 3 B (right panels; equal counts of each).

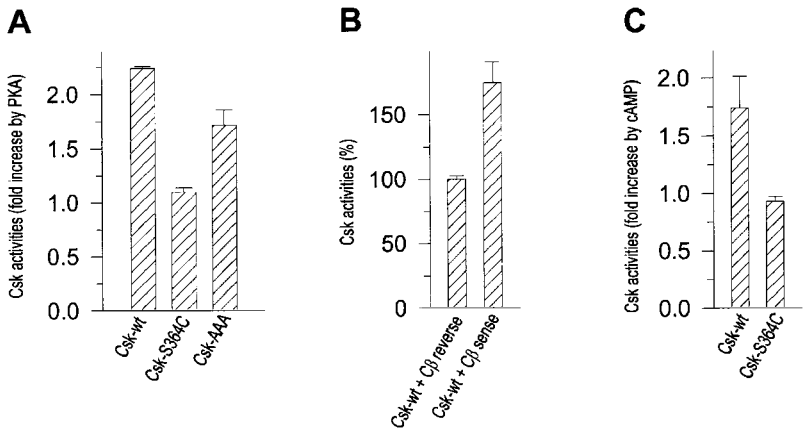

Figure 6. PKA phosphorylation of Csk-S364 is necessary for the regulatory effect of cAMP on Csk. (A) In vitro Csk kinase activities of Csk-wt, Csk-S364C, and Csk-S339A/S340A/T341A (Csk-AAA) were examined in the presence of active or heat-inactivated PKA Cα. (B) Csk kinase activity in anti-HA immunoprecipitates from Jurkat TAg T cells cotransfected with HA-Csk-wt and PKA Cβ subunit inserted in pEFneo in sense or reverse orientation. Kinase activities were normalized for levels of immunoreactive HA-Csk (means ± SEM). Expression of Cβ was also verified by immunoblotting. The data are representative of three independent experiments. (C) Csk activity was assessed in anti-HA immunoprecipitates of Jurkat TAg T cells transfected with HA-Csk-wt or mutant HA-Csk-S364C (means ± SEM). Cells were incubated in the presence or absence of 8-CPT-cAMP (300 μM for 40 min), and immunoprecipitations and kinase assays were performed as in B. Western blot analysis confirmed equal levels of Csk expression.

Csk-S364C, was observed in vitro (Fig. 6 A). Coexpression of wild-type Csk with PKA Cβ showed a 1.8-fold increase in Csk activity compared with Jurkat TAg T cells transfected with the Csk construct together with a vector with Cβ in the reverse orientation (Fig. 6 B). However, in contrast to the 1.8-fold increase in activity of wild-type Csk by treatment of Jurkat T cells with cAMP, the activity of the mutant Csk-S364C enzyme was not affected by cAMP (Fig. 6 C).

A PKA-Csk-Lck Inhibitory Pathway Mediates cAMP Regulation of IL-2 Production. To assess the downstream effects of PKA-mediated activation of Csk on T cell activation, we examined TCR-induced IL-2 production in Jurkat T cells (clone E6.1). To avoid dilution by untransfected cells, we developed a protocol for the selection of transfected cells. Cotransfection with DNA encoding the rat NK cell receptor NKR-P1A and magnetic bead selection for receptor allowed purification of cells expressing green fluorescent protein (Fig. 7 A) or Csk (Fig. 7 B). TCR-induced IL-2 production was very sensitive to the levels of expressed Csk, and a 3.5-fold overexpression reduced IL-2 secretion almost down to basal levels (Fig. 7 C). Thus, although the relative effect of cAMP was constant, the magnitude of the inhibition by cAMP was strongly reduced at higher levels of Csk expression (Fig. 7 C, ○). The effect of mutagenesis of S364 in Csk on the cAMP-inhibitable IL-2 production was therefore analyzed at a 1.9:1 ratio of transfected over endogenous Csk (arrow in Fig. 7 C) where changes in the inhibition by cAMP could be measured readily. Expression of Csk-S364C which has no PKA-phosphorylation site, reduced the cAMP inhibition of IL-2 production compared with control or cells expressing wild-type Csk (Fig. 7 D; 30 vs. 50–60% inhibition). The presence of endogenous Csk (1:1.9 versus mutant) explains why the inhibitory effect of cAMP was not totally abrogated. Higher levels of Csk-S364C expression by itself totally inhibited TCR-induced IL-2 production, and the effect of cAMP could not be analyzed. In contrast, Lck overexpression (twofold) by itself did not inhibit IL-2 production, which was fully sensitive to cAMP inhibition. However, Lck-Y505F strongly reduced the inhibitory effects of cAMP on TCR-induced IL-2 production (Fig. 7 E).

The PKA Type I-Csk-Lck Inhibitory Pathway Is Assembled in Lipid Rafts. We have reported previously the localization of PKA type I with the capped and activated TCR–CD3 complex (17). More recently, the understanding has been developed that proximal signaling events downstream of the TCR occur in specialized cholesterol- and glycolipid-enriched membrane microdomains or lipid rafts where signaling molecules such as Lck and LAT are targeted (27, 31). The novel lipid raft–associated Cpb/PAG is shown to interact with Csk in rat brain and in T cells via phosphotyrosine 317 in human PAG (Y314 in rat Cbp; references 9, 10). To analyze the subcellular distribution of components of the novel PKA-Csk-Lck inhibitory pathway mapped here, we purified lipid rafts by sucrose gradient centrifugation and fractionation of Triton X-100 lysates of peripheral blood T cells. Pervanadate treatment of T cells induced a strong tyrosine phosphorylation of the constitutively lipid raft–associated LAT, and increased the phosphotyrosine content of Cbp/PAG and Lck (Fig. 8 A) as well as other proteins not associated with lipid rafts (Fig. 8 A, lanes 9–12). However, both Cbp/PAG and Lck were phosphorylated also in resting peripheral T cells (Fig. 8 A, top). Furthermore, analysis of the same fractions showed that Csk, PKA RIα, and PKA C subunit are present in lipid rafts of both activated (Fig. 8 B) and resting (data not shown) T cells. In contrast, PKA RIIα is not detected in rafts, consistent with our earlier observations showing that PKA type I ($RI\alpha_2C_2$), and not PKA type II ($RII\alpha_2C_2$), mediates the inhibitory effect of cAMP on T cell immune function (16–18). Targeting of PKA type I may be mediated by an A-kinase anchoring protein (AKAP; for a review, see reference 32) directed to the RIα subunit and/or by docking of the C subunit e.g., via a caveolin-like protein (33). The constitutive association of Csk with rafts is consistent with the level of tyrosine phosphorylation of Cbp/PAG in resting T cells.

To functionally analyze the effect of PKA on Csk in rafts, we looked at Lck-defective JCaM1 T cells transfected

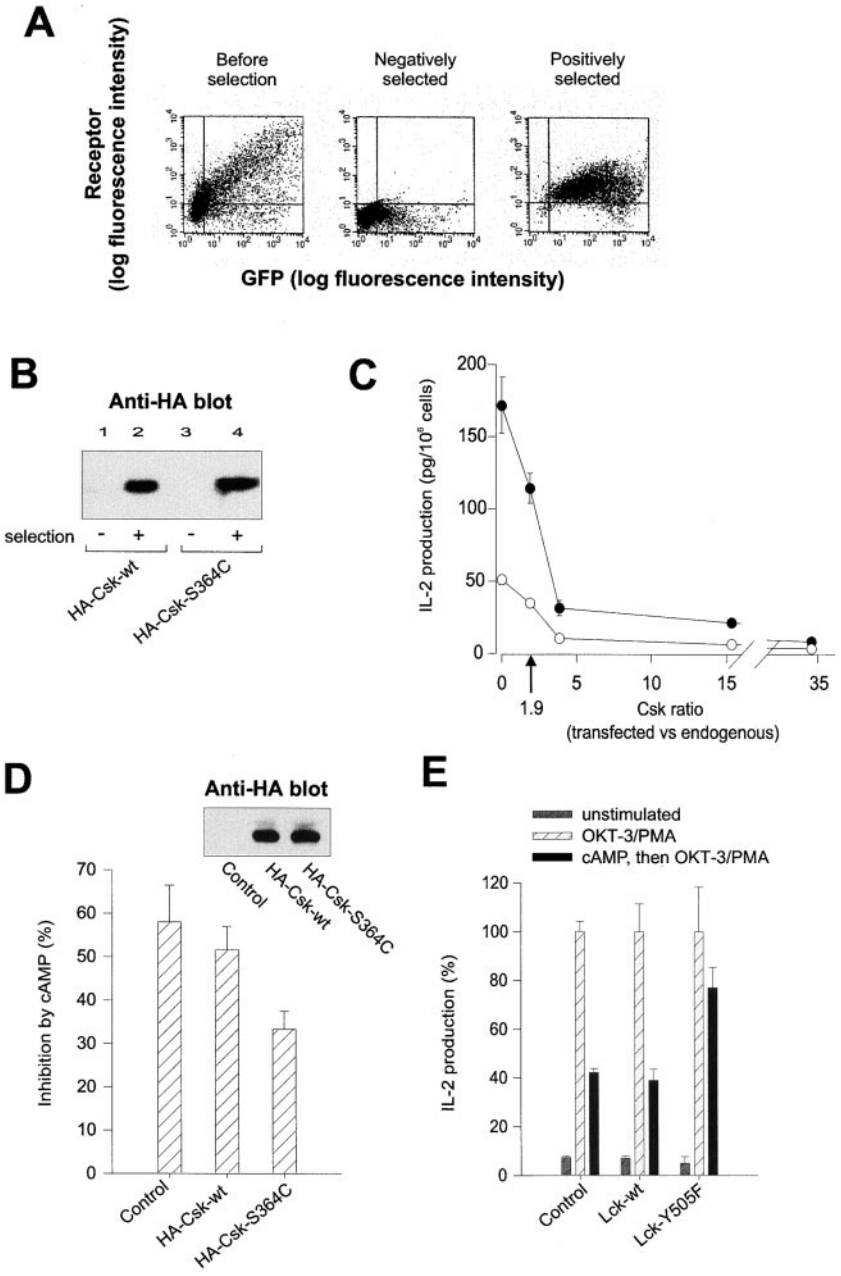

Figure 7. Pertubation of a PKA-Csk-Lck regulatory pathway mediates the inhibitory effect of cAMP on IL-2 secretion after T cell activation. (A) To establish a method for selecting only cells transfected with the gene of interest, Jurkat T cells were cotransfected with plasmids directing expression of a rat NK marker, NKR-P1A, and green fluorescent protein (GFP; left panel; 60% transfection efficiency, 47% double-positive cells), followed by separation of NKR-P1A positive (right) from negative (middle) cells using anti–NKR-P1 mAb and beads that allow detachment of cells after purification. Purity of the positive population was routinely 90–97%. (B) Anti-HA blot of cells transfected with HA-tagged Csk expression vectors and purified by bead selection for the cotransfected NKR-P1A. Negatively (−; lanes 1 and 3) and positively selected (+; lanes 2 and 4) cells are shown. (C) Jurkat T cells (clone E6.1) were transfected with Csk-wt expression vector at increasing doses of DNA, and the following day cells (6×10^5, 0.3×10^6/ml) positively selected for cotransfectant were stimulated by OKT3 (5 μg/ml) and PMA (10 nM) in the absence (●) or presence (○) of 8-CPT-cAMP (500 μM, 15 min pretreatment). After 20 h of culture, supernatants were harvested and analyzed for secreted IL-2. Pelleted cells were subjected to anti-Csk immunoblotting and densitometric scanning of levels of immunoreactive native and transfected (running with somewhat lower mobility) Csk. IL-2 levels were plotted relative to the ratio of transfected over native Csk. Arrow indicates the level of Csk expression used for the experiment in D. Representative of two experiments. (D) Positively selected Jurkat T cells expressing HA-Csk-wt or mutant HA-Csk-S364C at twofold above endogenous levels of Csk were stimulated and analyzed together with vector-transfected cells as in C for IL-2 secretion in the absence and presence of 8-CPT-cAMP. Percent inhibition of IL-2 secretion by cAMP for each cell culture is shown. In cells transfected with HA-Csk-wt, the total IL-2 production is inhibited both in the presence and absence of cAMP, whereas the ratio remains constant. Inset shows equal levels of transfected Csk-wt and Csk-S364C by anti-HA immunoblotting. Specific activities of wild-type and mutant Csk-S364C were comparable (data not shown). Representative of three experiments. (E) Jurkat T cells (five to six individual cell cultures) were transfected with empty vector (Control) or vectors directing expression of wild-type Lck and mutant Lck-Y505F that cannot be phosphorylated by Csk, stimulated as in C, and analyzed for IL-2 secretion (levels relative to stimulated are shown).

with kinase-dead Lck (Lck-K273M) that cannot be autophosphorylated at Y394 and therefore can only be tyrosine phosphorylated at Y505 (by Csk). Both transfected Lck and endogenous Csk were present in lipid rafts of these cells. Furthermore, when transfected cells were incubated in the presence of forskolin (to stimulate cAMP production), the tyrosine phosphorylation of Lck-K273M isolated from rafts increased 2.3-fold, indicating that Csk activity in rafts was stimulated upon triggering of the cAMP-PKA pathway (Fig. 9). Similar observations were made in whole cell lysates (data not shown).

Discussion

Csk is present in all human cells as a key regulator of Src kinases (7). The fact that the presence of Y505 in Lck is essential for the inhibitory effect of cAMP on ζ chain phosphorylation and IL-2 production indicates that the PKA-mediated phosphorylation of Csk may be a major mechanism by which cAMP inhibits TCR-mediated T cell activation (Fig. 10). A two- to fourfold increase in Csk activity by phosphorylation of S364 appears to have similarly distinct effects on T cell function as a two- to threefold Csk overexpression, which abolishes activation through the TCR (6)

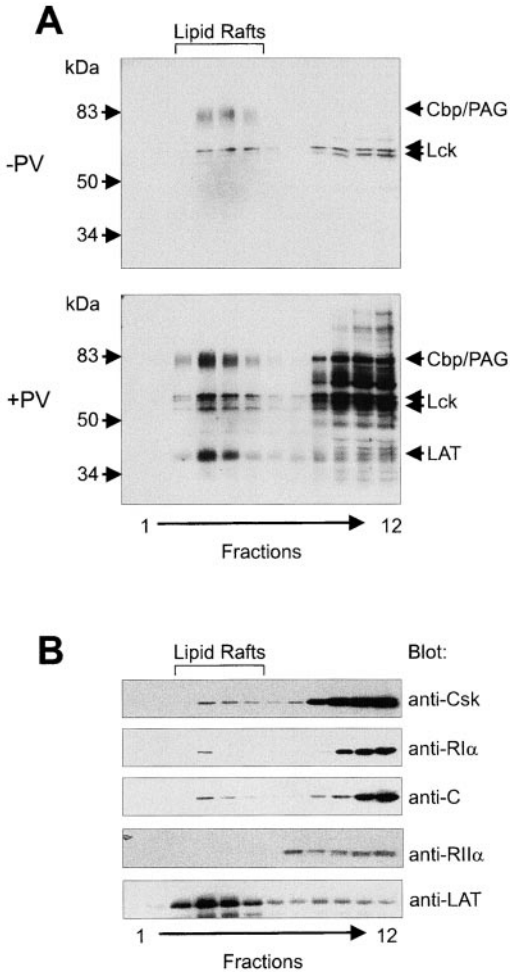

Figure 8. PKA and Csk are targeted to lipid rafts. Peripheral blood T cells were left untreated (−PV) or treated with pervanadate for 5 min (+PV) before homogenization in ice-cold lysis buffer with 1% Triton X-100 and separation in a 40–5% sucrose gradient. Fractions collected from the top (1–12) were analyzed by immunoblotting for (A) phosphotyrosine content and (B) distribution of Csk, PKA R and C subunits, and LAT. Mobility of molecular weight markers as well as of Cbp/PAG, Lck, and LAT are indicated in A. Blots in B represent parallel gel runs of the fractions in A (+PV). Observations are representative of three or more experiments.

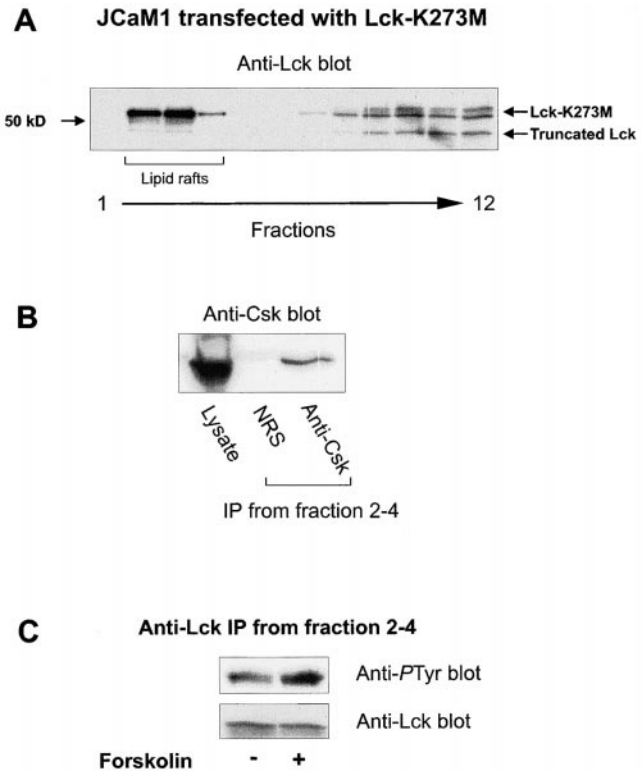

Figure 9. Forskolin stimulation of cells increases the phosphorylation of Y505 in Lck in lipid rafts. (A) Lck-deficient JCaM1 cells were transfected with a plasmid encoding catalytically inactive Lck-K273M. After harvesting, cells were homogenized in lysis buffer containing 0.7% Triton X-100 and subsequently separated in a 40–5% sucrose gradient. Fractions collected from the top (1–12) were analyzed by anti-Lck immunoblotting. Fractions 2–4 represent lipid raft fractions. Both transfected Lck-K273M and truncated catalytically inactive endogenous Lck are indicated. (B) Lipid raft fractions (fractions 2–4) from A were mixed and solubilized by addition of octyl-glucoside (50 mM). Thereafter, immunoprecipitation (IP) with either normal rabbit serum (NRS) or anti-Csk Abs was performed, and subsequent SDS-PAGE and anti-Csk immunoblotting were conducted. Triton X-100 lysate of JCaM1 cells is shown as control. (C) JCaM1 cells transfected with a plasmid encoding catalytically inactive Lck-K273M (same cells as in A) were incubated in the absence (−) or presence (+) of forskolin (100 μM) at 37°C for 10 min; thereafter lipid raft purification was performed as in A. Lipid raft fractions (2–4) were mixed and solubilized by the addition of octyl-glucoside (50 mM), and subjected to anti-Lck immunoprecipitation. After SDS-PAGE, the phosphotyrosine content of Lck was assessed by immunoblotting with antiphosphotyrosine Abs (4G10). Anti-Lck immunoblot (bottom) is shown as control. Densitometric analysis of both blots was conducted to assess the level of tyrosine phosphorylation of Lck-K273M.

and downstream IL-2 production (Fig. 7 C). Furthermore, the stoichiometry of Csk phosphorylation by PKA in vitro is 0.3–0.5 mol/mol of Csk under optimal conditions, indicating a single site not fully phosphorylated (quite common with bacterially produced protein). In vivo, we anticipate that a specific pool of Csk may be preferentially phosphorylated by colocalized PKA and reaches a higher stoichiometry and extent of activation. In addition, Jurkat and other leukemic T cell lines have higher levels of tyrosine phosphorylation (34) and Src kinase activities (35) than peripheral T cells. Thus, normal T cells appear to have a more controlled and managed Lck activity that may implicate Csk regulation and a PKA-Csk inhibitory pathway to a larger extent than apparent from, for example transfection studies on Jurkat T cells. Indeed, low level labeling of peptide 1 (representing Csk-S364) was seen in the tryptic peptide mapping of Csk from metabolically labeled unstimulated cells (Fig. 5 C) which increased strongly by treatment with PGE1 alone (data not shown). This indicates that this site is phosphorylated under physiological conditions. The mechanism for Csk activation by S364 phosphorylation is currently under investigation in our laboratory, and data in progress indicate that interaction with the intrachain SH3 domain is implicated in the PKA-mediated activation of Csk.

We have recently reported that the T cell dysfunction in HIV can be reversed by inhibition of the increased activity

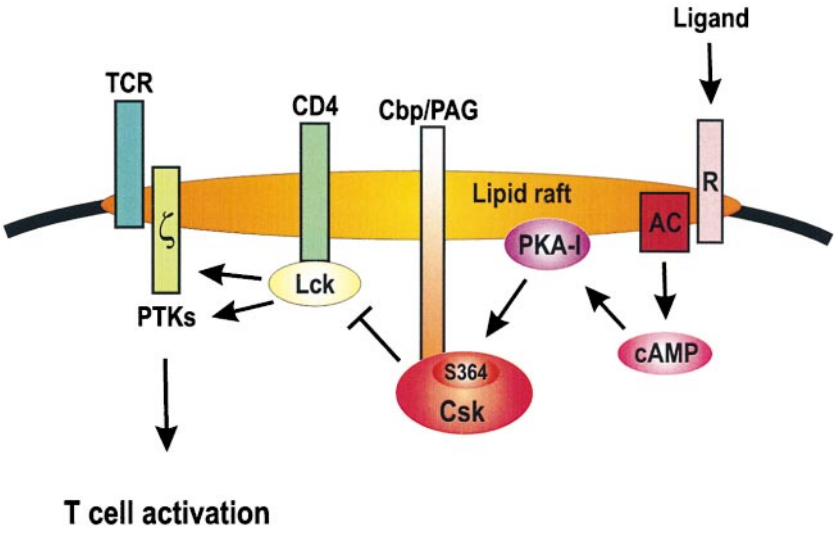

Figure 10. Model for a molecular mechanism whereby cAMP inhibits T cell function. Activation of adenylyl cyclase (AC), e.g., by binding of ligands such as adrenalin and PGE2 to G protein–coupled receptors (R), turns on a regulatory pathway assembled in lipid rafts that involves phosphorylation of Csk at S364 by PKA type I leading to activation of Csk. Activated Csk then phosphorylates and turns off Lck, and T cell activation is inhibited by the absence of ζ chain phosphorylation. Lipid raft association of Csk through interaction with phosphotyrosine Y314 in Cbp/Y317 in PAG and of PKA by interaction with A-kinase anchoring proteins and/or other docking proteins spatially facilitates modulation of T cell activation processes in lipid rafts by this inhibitory pathway.

of PKA type I (18), indicating that immunomodulation through cAMP/PKA contributes to the pathogenesis of this immunodeficiency. Inhibition of Lck through activation of Csk provides a molecular mechanism for this effect. Furthermore, PKA-mediated regulation of the activity of various Src kinase family members by phosphorylation of Csk may also provide a molecular mechanism for cAMP-mediated regulation of both B and NK cell activation (36, 37). Finally, Csk and Src kinases are expressed in other tissues, including neuronal tissues (38), and the impact of cAMP regulation of Csk in these tissues will be interesting to pursue. The PKA phosphorylation site in Csk is conserved between vertebrates, suggesting that this site may have been subject to selection pressure, but is only partially conserved (RFS or KFT) in Csk homologous kinase (Chk/Lsk/Hyl/Matk) and Csk-type protein kinase (Ctk/Bhk/Ntk).

In conclusion, we report the mapping of a PKA phosphorylation site on Csk and regulation of Csk activity by cAMP/PKA. Localization of both Csk and PKA type I to lipid rafts supports the notion that this novel inhibitory pathway is assembled in membrane microdomains where it can intersect TCR-induced signaling at a proximal level. The presence of adenylyl cyclase that generates cAMP in lipid rafts of S49 lymphoma cells further supports assembly of the cAMP-PKA type I-Csk inhibitory pathway in lipid rafts (Fig. 10; reference 39). The constitutive localization of components of this pathway in lipid rafts may indicate that a tonic level of inhibition of T cell activation is imposed on resting T cells. PKA-mediated activation of Csk provides a molecular mechanism for cAMP-dependent inhibition of lymphocyte activation, and Csk-S364 and/or Lck-Y505 may be future targets for immunomodulating therapies.[2] Furthermore, this mechanism may regulate signaling through Src kinases in general.

[2]Targeting of PKA type I, Csk, and Lck-Y505 for treatment of immunodeficiencies is described in pending patent applications, no. W098148809 and no. W099162315 with priority from April 20, 1997 and May 27, 1998, respectively.

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