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Combined Spatial and Enzymatic Regulation of Csk by cAMP and Protein Kinase A Inhibits T Cell Receptor Signaling*

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Raft-associated Csk controls signaling through the T cell receptor (TCR) and was mainly anchored to Cbp/PAG (phosphoprotein associated with glycosphingolipid-enriched membrane domains). Treatment of cells with the cAMP-elevating agent prostaglandin E_2 (PGE_2) augmented the level of Cbp/PAG phosphorylation with a concomitant increase in amounts of Csk bound to Cbp/PAG. While TCR-triggering resulted in transient dissociation of Csk from Cbp/PAG/rafts allowing TCR-induced tyrosine phosphorylation to occur, pretreatment with PGE_2 reduced Csk dissociation upon TCR triggering. This correlated with lowered TCR-induced phosphorylation of CD3 ζ-chain and linker for activation of T cells. Moreover, competition of endogenous Csk from lipid rafts abolished PGE_2-mediated inhibition of TCR-induced ζ-chain phosphorylation and activation of the nuclear factor of activated T cells (NFAT) activator protein 1 (AP-1). Finally, raft-associated Csk already activated via Cbp/PAG binding, gained additional increase in phosphotransferase activity upon protein kinase A-mediated phosphorylation of Csk. We propose that cAMP regulates Csk via both spatial and enzymatic mechanisms, thereby inhibiting signaling through the TCR.

Upon triggering of the T cell receptor (TCR)[1] activation of Lck leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs of the CD3 complex (1). The physiologically relevant pool of Lck most probably partitions into lipid rafts (2). In resting T cells this pool of Lck is kept in an inactive state due to phosphorylation of Tyr^{505} by Csk, which is recruited to rafts via binding of its SH2 domain to phosphorylated Tyr^{317} in the transmembrane adaptor molecule Cbp/PAG (2–4). Upon TCR triggering, Csk transiently dissociates from rafts, allowing Lck to become activated (4, 5). Thus, tonic repression of Lck activity in rafts by Csk seems to set the threshold for TCR signaling and appears necessary to avoid unregulated TCR signaling and activation. This model is supported by several observations: (i) modest overexpression (2.5–3.2-fold) or membrane targeting of Csk inhibit TCR-induced tyrosine phosphorylation and IL-2 production (6), (ii) studies on inducible *csk* knock-outs revealed up-regulated Lck/Fyn activities (7), (iii) displacement of endogenous Csk from lipid rafts has stimulatory effects on ζ-chain phosphorylation and IL-2 promoter activation both in resting T cells and after TCR triggering (5). Thus, control of Csk activity in rafts seems to be of major importance to prevent aberrant TCR signaling.

Cyclic AMP (cAMP) inhibits TCR-induced T cell activation and thereby exerts important immunoregulatory functions (8). Based on studies with selective agonists, activation of protein kinase A (PKA) type I is necessary and sufficient for mediating these effects of cAMP (9). We recently found that PKA through phosphorylation of Ser^{364} in Csk induces a 2–4-fold activation of Csk in T cell lipid rafts (10), making Csk the most upstream PKA target reported so far (reviewed in Ref. 11). However, the question of how PKA can regulate Csk if the latter dissociates from rafts upon TCR triggering remained to be addressed. Here, we report that prostaglandin E_2 (PGE_2) and cAMP induce increased targeting of Csk to rafts, where Csk is further activated by PKA-mediated phosphorylation. This dual effect on Csk localization and activity by cAMP inhibits signaling through the TCR.

EXPERIMENTAL PROCEDURES

T Cell Purification and Transfection—Human peripheral blood T cells were purified by negative selection as described previously (12), transfected in accordance with the manufacturer's instructions by using the Amaxa nucleofector and kit (catalog number VPA-1002); transfection efficiencies of more than 80% were achieved. Jurkat TAg cells were transfected as described previously (5).

Reagents and Antibodies—PGE_2 and protein kinase A inhibitor peptide (catalog number P-6062) were purchased from Sigma, *n*-octyl-β-D-glucoside from United States Biochemical, and forskolin from Calbiochem. Phospho- and dephospho-Tyr^{317}-PAG peptides (from Eurogentec) are 10-mer peptides corresponding to the sequence surrounding Tyr^{317} in Cbp/PAG and have been described earlier (13). All antibodies were as described previously (5, 10). A standard cAMP assay (kit from PerkinElmer Life Sciences, catalog number SP004) was performed in accordance with the manufacturer's instructions. The program Scion Image from Scion Corp. was used for densitometric scanning analysis.

Constructs—The different Csk constructs have been described elsewhere (5). An N-terminally HA-tagged chimeric LAT36-protein kinase A inhibitor (PKI) construct in pEF-HA-Bos vector consisted of the 36 N-terminal amino acids of human LAT (this includes the extracellular and transmembrane domains and the membrane proximal part of the intracellular portion including cysteine residues important for lipid raft localization) fused to PKI. Another LAT-PKI chimer consisted of the N-terminal 39 amino acids of rat LAT fused to PKI (called LAT39-PKI),

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[1] The abbreviations used are: TCR, T cell antigen receptor; PGE_2, prostaglandin E_2; PKA, protein kinase A; PKI, protein kinase inhibitor; Csk, C-terminal Src kinase; Cbp, Csk-binding protein; PAG, phosphoprotein associated with glycosphingolipid-enriched membrane domains; wt, wild type; HA, hemagglutinin epitope; IL, interleukin; SH, Src homology; LAT, linker for activation of T cells; NFAT, nuclear factor of activated T cells; AP-1, activator protein 1.

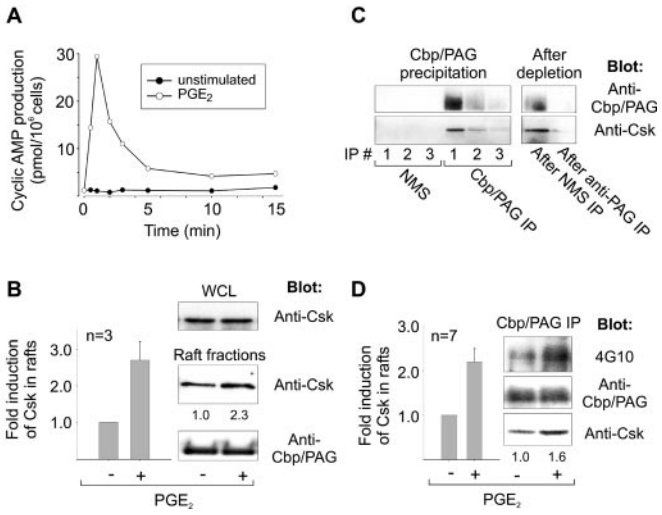

FIG. 1. **Cyclic AMP increases interaction between Csk and Cbp/PAG.** *A*, T cells were incubated with PGE_2 (2 μM) for the indicated times and levels of cAMP were measured. *B*, normal T cells were incubated with or without PGE_2 (100 μM) for 1.5 min. After lysis of cells and sucrose-gradient fractionation, the lipid raft containing fractions were mixed and immunoblotted with the indicated antibodies (*middle* and *lower panels*). The *numbers* below the *middle panel* (Csk blot) are results from densitometric scanning analysis. *Bars* represent results from several experiments (average ± S.E.). Whole cell lysates were also assessed with respect to Csk content (*upper panel*). *C*, purified lipid raft samples from normal T cells were subjected to three consecutive rounds of immunoprecipitation with either anti-Cbp/PAG antibody or non-immune mouse serum (*NMS*), and samples were then immunoblotted for Cbp/PAG and Csk. The immunoprecipitates were immunoblotted for Cbp/PAG and co-precipitated Csk. *D*, primary T cells were stimulated with or without PGE_2 as in *B*. After disruption of cells in lysis buffer (with *n*-octyl-β-D-glucoside), Cbp/PAG immunoprecipitates were analyzed with the indicated antibodies as described in the legend for *B*.

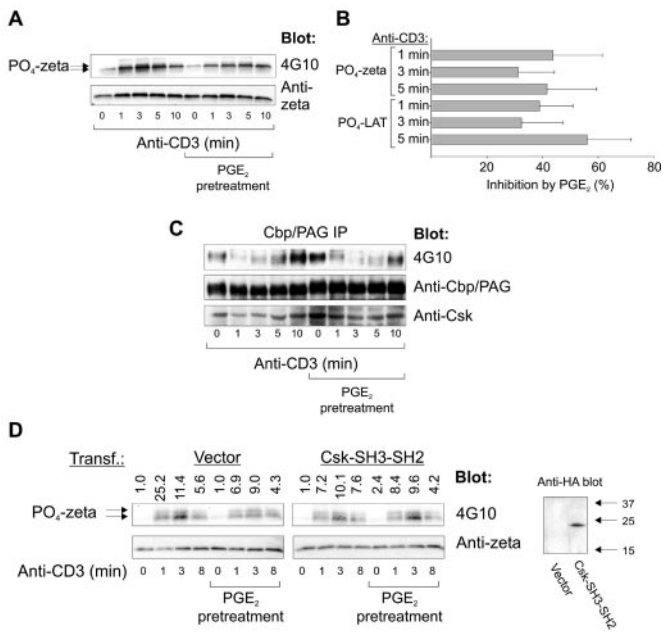

FIG. 2. **PGE_2-mediated regulation of raft-associated Csk represses TCR-induced tyrosine phosphorylation.** *A*, normal T cells were incubated with or without PGE_2 for 1.5 min, then OKT-3 was added and samples at time 0 withdrawn and disrupted in lysis buffer containing *n*-octyl-β-D-glucoside. 2 min later $F(ab')_2$ fragments were added to allow TCR cross-linking, and samples withdrawn at the indicated periods of time (called anti-CD3). Lysates were immunoblotted with the indicated antibodies. *B*, data from four experiments as in A were analyzed with respect to total phosphotyrosine content in CD3 ζ (p21 and p23) and LAT and normalized for the amount of p16 (CD3 ζ). The inhibition in TCR-induced tyrosine phosphorylation caused by PGE_2 pretreatment is given. *C*, same as described in the legend for *A*, but after disruption of cells, Cbp/PAG immunoprecipitates were analyzed with the indicated antibodies. *D*, primary T cells were transfected with either empty vector or a plasmid encoding kinase-deficient HA-tagged Csk-SH3-SH2, and the following day an experiment as described in the legend for *A* was conducted. The *numbers* on *top* of the 4G10 blots are results from densitometric scanning analysis (ratio between total ζ-chain phosphorylation and p16 of ζ; values are relative to the ratio for unstimulated cells). The two 4G10 blots are derived from different gels and should therefore only be compared qualitatively. Expression of the HA-tagged Csk construct is also shown.

where the tyrosine residue in the cytoplasmic region of LAT had been mutated to phenylalanine by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA).

Stimulation of Cells, Purification of Lipid Rafts, Immunoprecipitations, and Kinase Assays—T cells were stimulated or not with PGE_2 (100 μM for 1.5 min if not stated otherwise); thereafter, anti-CD3 ϵ monoclonal antibody OKT-3 (5 μg/ml) was added and 2 min later CD3 was cross-ligated by addition of $F(ab')_2$ fragments (20 μg/ml), and incubations were continued. Cells were disrupted in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1.0% Triton X-100 with 2 mM sodium orthovanadate, 1 mm phenylmethylsulfonyl fluoride, 10 mm sodium pyrophosphate, and 50 mm sodium fluoride) containing *n*-octyl-β-D-glucoside (50 mM) and subjected to immunoprecipitation, as described previously (10). Lipid raft fractions were isolated as before from cell lysates (with 1% Triton X-100) by sucrose-gradient centrifugation (5). In the presence of ATP (1 mM) and $MgCl_2$ (15 mM) lipid raft fractions were stimulated with or without forskolin (100 μM, 10 min) or PGE_2 (100 μM, 2 min) at 30 °C; thereafter, reactions were stopped by addition of lysis buffer containing *n*-octyl-β-D-glucoside, and subsequently Csk immunoprecipitation and Csk kinase assay with poly(Glu,Tyr) as substrate were performed as described previously (10). Equal amounts of immunoprecipitated Csk or co-immunoprecipitated Cbp/PAG present in each kinase reaction were verified by immunoblotting. Cloning, expression, and purification of human Csk, Csk-S364C, and PKA Cα have been reported previously (10).

IL-2 Promoter Activity and T Cell Proliferation Assay—These assays were conducted as described previously (5, 12).

RESULTS AND DISCUSSION

Csk Interaction with Cbp/PAG Increases following cAMP Treatment—PGE_2 (2 μM) elicited a rapid and robust cAMP response in T cells (Fig. 1*A*), whereas maximal cAMP responses were observed at 15–50-fold higher concentrations of PGE_2 (30–100 μM) (data not shown). Interestingly, the total amount of Csk present in lipid rafts of resting T cells increased upon PGE_2 stimulation (densitometric scanning analysis: 2.7-fold increase ± 0.5, average ± S.E., $n = 3$), whereas neither the level of raft-associated Cbp/PAG nor the total amount of Csk present in whole cell lysates were changed (Fig. 1*B*). In T cells Cbp/PAG is exclusively present in lipid rafts (data not shown), and immunodepletion of Cbp/PAG from isolated rafts removed the majority of raft-associated Csk (Fig. 1*C*), indicating that Cbp/PAG is the main protein recruiting Csk to rafts in T cells. PGE_2 stimulation of resting T cells resulted in an increase in Cbp/PAG tyrosine phosphorylation status, and more importantly, a concomitant increase (densitometric scanning analysis: 2.2-fold ± 0.3, average ± S.E., $n = 7$) in the amounts of Csk that co-immunoprecipitated with Cbp/PAG (Figs. 1*D* and 2*B*). Similar findings were also obtained with forskolin (not shown). Altogether, this points toward a role for cAMP in modulating the total amount of Csk in lipid rafts of resting T cells.

Inhibition of TCR-induced Signaling by cAMP Is Dependent on Raft-associated Csk—We next tested whether elevated amounts of Csk in rafts induced by cAMP could repress signaling through the TCR. Incubation of T cells with PGE_2 prior to TCR triggering inhibited TCR-induced tyrosine phosphorylation of ζ-chain (Fig. 2, *A* and *B*), LAT (Fig. 2*B*), and Slp-76 (not shown). While TCR triggering resulted in transient dephosphorylation of Cbp/PAG and dissociation of Csk from Cbp/PAG (and thereby rafts), preincubation of PGE_2 prior to TCR stimulation increased the amount of Csk co-immunoprecipitating with Cbp/PAG both in the resting state and the first minutes

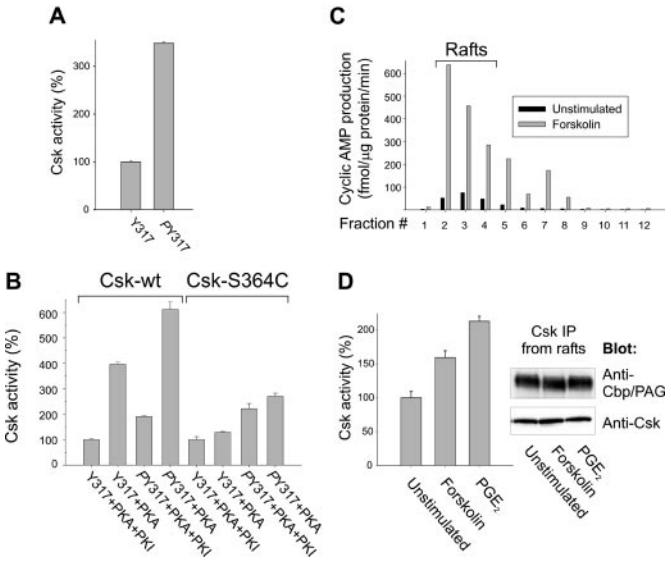

FIG. 3. **Csk activated by engagement of its SH2 domain can be further activated by PKA-mediated phosphorylation.** *A*, the phosphotransferase activity of recombinant Csk-wt (1 ng/μl) toward the synthetic polyamino acid poly(Glu,Tyr) was measured in the presence of either phospho-Tyr^{317}-PAG peptide (*PY317*) or dephospho-Tyr^{317}-PAG peptide (*Y317*) (10 μg/ml). *B*, the phosphotransferase activity of recombinant Csk-wt or Csk-S364C was assessed as described in the legend for *A* in the presence of PKA C subunit (1 ng of active C/μl) with or without PKI (200 μg/ml) and Tyr^{317}/Tyr(P)^{317} PAG peptides (10 μg/ml). *C*, isolated lipid raft fractions from Jurkat T cells were reconstituted with Mg-ATP and stimulated with or without forskolin (100 μM, 3 min) at 30 °C, then cAMP levels were measured. *D*, isolated lipid raft fractions from normal T cells were mixed and reconstituted with Mg-ATP and stimulated with or without forskolin or PGE_2. Thereafter reactions were stopped by addition of ice-cold lysis buffer (with *n*-octyl-β-D-glucoside), and phosphotransferase activity of immunoprecipitated Csk was assessed as described in the legend for *A*. Equal amounts of immunoprecipitated Csk or co-immunoprecipitated Cbp/PAG present in each kinase reaction were verified by immunoblotting, and one typical blot is shown. Data represent the average (mean ± S.D.) of six independent reactions

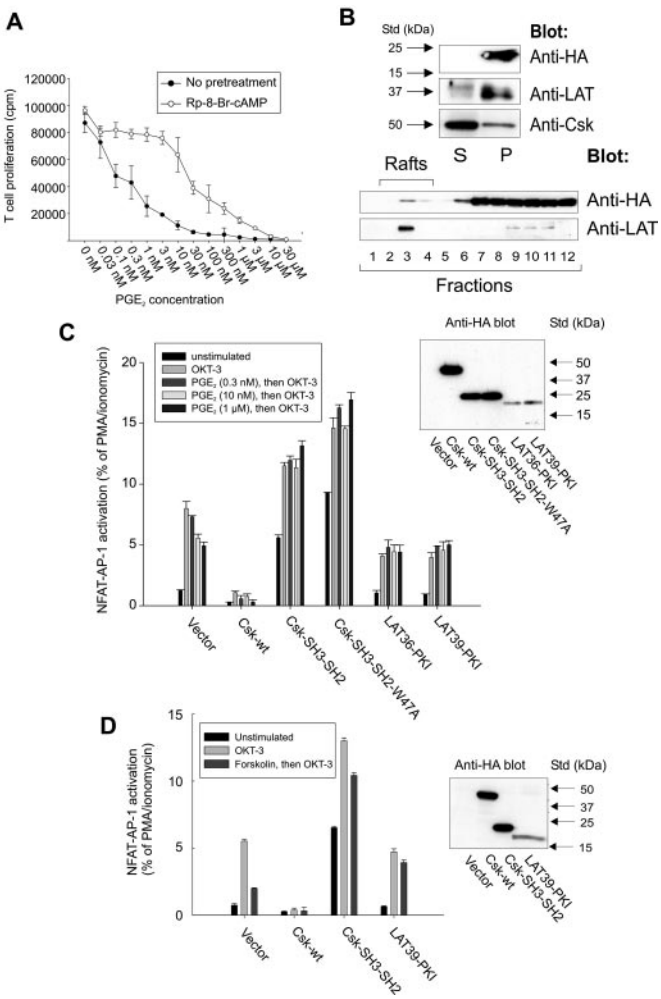

FIG. 4. **Lipid raft-associated Csk is essential for the inhibitory effect of cAMP on TCR-induced NFAT-AP-1 activation.** *A*, the effects of different PGE_2 concentrations on TCR-induced proliferation of purified T cells were assessed as [^{3}H]thymidine incorporation with or without preincubation of the cAMP-antagonist Rp-8-bromo-cAMP (30 min, 1000 μM). *B*, Jurkat TAg cells were transfected with a plasmid encoding HA-tagged LAT-PKI. The next day the amounts of HA-tagged LAT-PKI present in soluble (*S*) and particulate (*P*) fractions were assessed (*upper panel*). In addition, sucrose-gradient fractionation of transfected cells was also performed (*lower panel*). The LAT and Csk blots serve as controls. *C*, Jurkat TAg cells were co-transfected with NFAT-AP1-luciferase reporter construct and plasmids encoding either different Csk constructs or LAT-PKI chimeras. The following day, cells were incubated with OKT-3 with or without PGE_2 pretreatment (different concentrations) or with PMA/ionomycin (25 ng/ml and 5 μM, respectively). After 6 h of incubation, luciferase activity was assessed. Expression control is also shown. *D*, same as described in the legend for *C*, but prior to OKT-3 stimulation cells were pretreated or not with forskolin (100 μM, 10 min).

after TCR-triggering (Fig. 2*C*). We next transfected primary T cells with either empty vector or a plasmid encoding kinase-deficient Csk-SH3-SH2, which has intact and functional SH3 and SH2 domains but lacks the kinase domain. Overexpression of this latter construct has previously been shown to displace endogenous Csk from lipid rafts (5). While T cells transfected with empty vector revealed normal TCR-induced ζ-chain phosphorylation (and tyrosine phosphorylation of LAT and Slp-76, not shown) that was inhibited by PGE_2 preincubation, transfection of kinase-deficient Csk-SH3-SH2 abolished the inhibitory effects of PGE_2 (Fig. 2*D*). Similar findings were obtained with Jurkat TAg cells (not shown). Altogether, this indicates that the inhibitory effects of PGE_2/cAMP on TCR signaling are dependent on the presence of Csk in rafts and/or the ability of recruiting additional amounts of Csk to rafts.

Engagement of Csk SH2 Domain and PKA-mediated Phosphorylation of Csk-S364 Both Activate Csk but via Separate Mechanisms—Previous findings have demonstrated that engagement of the Csk SH2 domain by binding to phosphorylated Cbp/PAG leads to a 2–4-fold increase in Csk kinase activity (13). A similar induction of Csk activity is caused by PKA-mediated phosphorylation of Csk in rafts (10). Since cAMP elevates the total amount of raft-associated Csk via increased interaction between Csk and Cbp/PAG, we next tested the impact of the concerted action of Cbp/PAG-binding and PKA-mediated phosphorylation on Csk kinase activity. As reported earlier (13), co-incubation of purified Csk with a 10-mer peptide corresponding to phosphorylated Tyr^{317} in Cbp/PAG (called Tyr(P)^{317} peptide) resulted in an ~3-fold induction of Csk kinase activity compared with the effect of a corresponding dephospho peptide (called Tyr^{317} peptide) (Fig. 3*A*). As expected, co-incubation of the Tyr^{317} peptide with Csk and PKA catalytic subunit (Cα) resulted in a Csk kinase activity that was 4-fold higher than when PKI was added to the reaction mixture (Fig. 3*B*). Interestingly, co-incubation of Cα with Csk, which is activated through engagement of its SH2 domain with Tyr(P)^{317} peptide, revealed an additional increase in Csk activity compared with a similar reaction mixture where PKI was also present (Fig. 3*B*). In addition, Csk-S364C, which is mutated in the PKA phosphorylation site, could no longer be activated by PKA but was still activated through engagement of its SH2 domain with Tyr(P)^{317} peptide (Fig. 3*B*). Altogether this indi-

cates that with recombinant proteins *in vitro*, activation of Csk by engagement of the Csk SH2 domain and by PKA-mediated phosphorylation together induces a higher level of activation of Csk (6-fold) than each mechanism contributes separately. We next tested whether raft-associated Csk, which is bound to phosphorylated Cbp/PAG, could gain additional increase in kinase activity by PKA-mediated phosphorylation. We have previously shown that PKA C and RI subunits are present in lipid rafts from T cells (10). Furthermore, both forskolin (Fig. 3*C*) and PGE_2 (not shown) induced cAMP production when purified lipid rafts from Jurkat T cells were reconstituted with Mg-ATP. This means that all components necessary for ligand-induced, PKA-mediated phosphorylation of Csk are present in rafts. When purified lipid rafts from resting T cells were reconstituted with Mg-ATP, stimulation with either PGE_2 or forskolin increased the activity of immunoprecipitated Csk compared with control (Fig. 3*D*). Since the amounts of co-immunoprecipitated Cbp/PAG were equal in all the Csk immunoprecipitations assessed (Fig. 3*D*), the observed differences in Csk kinase activity were most probably due to PKA-mediated phosphorylation of Csk induced by the cAMP-elevating agents. Altogether, this indicates that Csk already activated via binding to Cbp/PAG in rafts can achieve an additional increase in kinase activity upon PKA-mediated phosphorylation. This also means that the specific phosphotransferase activity of PKA-phosphorylated, Cbp/PAG-bound Csk is 6–8-fold higher than that of cytosolic, unphosphorylated Csk.

The Inhibitory Effect of cAMP on TCR-induced NFAT-AP-1 Activation Is Dependent on Raft-associated Csk—Multiple molecular targets for cAMP/PKA have been defined in the signaling cascades downstream of the TCR (reviewed in Ref. 11). To test the physiological relevance of raft-associated Csk as a PKA target, we studied downstream signaling events in T cells. As expected, PGE_2 inhibited TCR-induced T cell proliferation in a concentration-dependent manner, while the additional presence of the cAMP-antagonist Rp-8-bromo-cAMP right-shifted the curve (Fig. 4*A*), implicating that the observed effects of PGE_2 are mediated by cAMP. We next performed transfection studies with plasmids encoding either kinase-deficient Csk-SH3-SH2 (or Csk-SH3-SH2-W47A, which also has a defect SH3 domain) or LAT-PKI chimeras consisting of the N-terminal part of LAT (including the lipid raft targeting domain) fused to PKI. The Csk mutants have the ability to displace endogenous Csk from lipid rafts, while the LAT-PKI chimeras showed membrane localization in immunofluoresence studies (not shown), distributed mainly to the particulate fraction (Fig. 4*B*, *upper panel*) and also partitioned into lipid raft fractions (Fig. 4*B*, *lower panel*). Thus, the LAT-PKI chimeras potentially could inhibit membrane-bound PKA activity. PGE_2 pretreatment inhibited TCR-induced NFAT-AP-1 activation in Jurkat TAg cells ~40% (Fig. 4*C*), while expression of kinase-deficient Csk-SH3-SH2 or the different LAT-PKI chimeras abolished these inhibitory effects of PGE_2 (Fig. 4*C*). Forskolin induced a stronger and more sustained cAMP response compared with PGE_2 (not shown) and yielded up to 65% inhibition of TCR-induced NFAT-AP-1 activation when control transfected cells were pretreated with forskolin (Fig. 4*D*). In contrast, cells transfected with either kinase-deficient Csk or LAT-PKI were almost insensitive to the inhibitory effects of forskolin (20 and 17% inhibition, respectively, Fig. 4*D*). Altogether, this indicates that both raft-associated Csk and membrane-bound PKA are essential for the inhibitory effects of PGE_2/cAMP on TCR-induced downstream signaling events such as NFAT-AP-1 activation.

The effects of cAMP/PKA on raft-associated Csk described in this paper, including both spatial and enzymatic regulation of Csk, result in repression of signaling through the TCR, and hence T cell activation. However, how cAMP induces increased phosphotyrosine levels in Cbp/PAG, and thereby facilitates Csk binding, is still an enigma and suggests the involvement of additional targets regulated by cAMP. This can encompass both protein-tyrosine kinases and protein-tyrosine phosphatases. Src family kinases are reported to phosphorylate Cbp/PAG (3, 4), but at least in T cells the activity of Lck is lowered upon cAMP-stimulation, most probably due to activation of Csk (10). Nevertheless, a recent report points toward an essential role for Fyn in the phosphorylation of Cbp/PAG in T cells (14). *In vitro* studies have revealed that Csk also can phosphorylate Cbp/PAG, but only when Cbp/PAG has a certain phosphotyrosine content beforehand (13). The protein-tyrosine phosphatases responsible for regulation of Cbp/PAG phosphorylation status are currently being unraveled, and a recent report implicated CD45 in dephosphorylation of Cbp/PAG upon TCR triggering (15). However, further studies appear necessary to identify the targets whereby cAMP regulates tyrosine phosphorylation of Cbp/PAG.

In conclusion, we report that the cAMP/PKA signal pathway through dual mechanisms of increased anchoring and direct phosphorylation-dependent activation regulates Csk, thereby inhibiting signaling through the TCR. This involves cAMP-driven recruitment of Csk to rafts where PKA-mediated phosphorylation of Csk results in elevated levels of raft-associated Csk phosphotransferase activity.

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