

# Feedback Circuits Monitor and Adjust Basal Lck-Dependent Events in T Cell Receptor Signaling

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The Src family kinase Lck is crucial for the initiation of TCR signaling. The activity of Lck is tightly controlled to prevent erroneous immune activation, yet it enables rapid cellular responses over a range of sensitivities to antigens. Here, in experiments with an analog-sensitive variant of the tyrosine kinase Csk, we report that Lck in T cells is dynamically controlled by an equilibrium between Csk and the tyrosine phosphatase CD45. By rapidly inhibiting Csk, we showed that changes in this equilibrium were sufficient to activate canonical TCR signaling pathways independently of ligand binding to the TCR. The activated signaling pathways showed sustained and enhanced phosphorylation compared to that in TCR-stimulated cells, revealing a feedback circuit that was sensitive to the basal signaling machinery. We identified the inhibitory adaptor molecule Dok-1 (downstream of kinase 1) as a candidate that may respond to alterations in basal signaling activity. Our results also suggest a role for Csk in the termination or dampening of TCR signals.

## INTRODUCTION

Cell surface receptors, such as the T cell receptor (TCR), are studied in the context of ligand activation and are controlled by a threshold of activation that is dependent on ligand affinity and avidity. TCR signaling is critical for the development, survival, and activation of mature lymphocytes, and TCR signal strength greatly influences the repertoire of TCRs on the T cells that populate the immune system (1, 2). Sufficient activation of TCR signaling is necessary for the differentiation of naïve T cells into effector and memory T cells during an immune response (3). Comparatively little work has focused on the basal state of the TCR before ligand binds. Here, we uncover an unexpected amount of basal TCR signaling in the absence of ligand, which suggests that the cytoplasmic signaling network downstream of the TCR is poised to rapidly respond, yet is restrained by a single inhibitory kinase.

The TCR complex contains no endogenous kinase function, but uses the Src family kinase (SFK) Lck to phosphorylate paired tyrosine residues in cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) in each of the CD3 and  $\zeta$  chains of the TCR complex (4). The tyrosine kinase C-terminal Src kinase (Csk) is a critical inhibitor of SFK activity and phosphorylates the conserved C-terminal inhibitory Tyr<sup>505</sup> in Lck. Phosphorylation of Lck Tyr<sup>505</sup> results in stabilization of an inactive conformation that inhibits the catalytic function of Lck and prevents its access to substrates (4). In T cells, Csk-mediated phosphorylation of Tyr<sup>505</sup> is functionally opposed by the receptor-like tyrosine phosphatase CD45, which dephosphorylates Tyr<sup>505</sup>, enabling Lck to phosphorylate ITAMs (5). In contrast to Tyr<sup>505</sup>, phosphorylation of the conserved Tyr<sup>394</sup> in the activation loop of the catalytic domain of Lck is associated with increased kinase activity, although some work suggests that TCR stimulation may not markedly alter the total extent of Tyr<sup>394</sup> phosphorylation (6).

Within the immune system, Csk is crucial for controlling lymphocyte development and preventing aberrant activation of immune cells. Csk is regulated primarily by its subcellular localization and by interactions with other proteins through its Src homology 2 (SH2) and SH3 domains. In unstimulated T cells, Csk is enriched in lipid raft fractions of the plasma membrane, the result of putative SH2-mediated interactions with lipid raft-enriched adaptor proteins, including phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (7, 8) and presumably other proteins. After TCR stimulation, PAG is rapidly dephosphorylated by an unknown mechanism, resulting in release of Csk into the cytoplasm. Dissociation of active Csk from the plasma membrane favors the action of CD45, thus promoting the activity of Lck and other SFKs (9). Because PAG-deficient T cells have no obvious phenotype, other as yet unknown membrane recruitment mechanisms for Csk are likely to exist.

The regulation of Lck is critical for orchestrating the threshold sensitivity and strength of TCR signaling; however, it remains unclear whether the activation state of Lck is “fixed” in resting T cells or is the result of a dynamic equilibrium between ongoing Csk and CD45 activities. In a fixed state, Lck activation would require specific changes in the localization or catalytic activities of its regulatory proteins, whereas a dynamic equilibrium between Csk and CD45 might continuously alter the phosphorylation status and activity of Lck. Hence, a small imbalance in the activities of either CD45 or Csk would be sufficient to alter Lck activity. Rapid perturbation of Csk function has been hampered because of the prolonged time needed to express exogenous alleles of mutant signaling proteins. No selective small-molecule inhibitor of Csk is available, because Csk inhibitors invariably also inhibit SFKs. Furthermore, *Csk*<sup>-/-</sup> mice suffer early embryonic lethality, and loss of Csk during early T cell development results in aberrant T cell development (10, 11).

To characterize the role of Csk in regulating basal and ligand-induced signaling downstream of the TCR, we established a small-molecule controlled Csk allele, *Csk*<sup>AS</sup>, that is capable of inhibiting TCR activation, particularly when *Csk*<sup>AS</sup> is localized to the plasma membrane. Rapid and specific inhibition of membrane-targeted *Csk*<sup>AS</sup> resulted in potent and sustained signal transduction and cell activation. This activation was independent of TCR ligation but used canonical TCR signaling components. We also suggest a previously uncharacterized role for Csk in the termination or dampening of the antigen receptor response. These findings reveal a

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control mechanism that is sensitive to the extent of basal signaling by the TCR pathway, which may feed back and alter the basal signal transduction machinery. Finally, we identify the inhibitory adaptor protein Dok-1 (downstream of kinase 1), which is involved in the membrane recruitment of Csk and inhibition of antigen receptor signaling in T cells (12), as a candidate component of the altered basal signaling circuitry.

## RESULTS

### Generation of the *Csk*<sup>AS</sup> T266G allele enables rapid and specific chemical inhibition of Csk

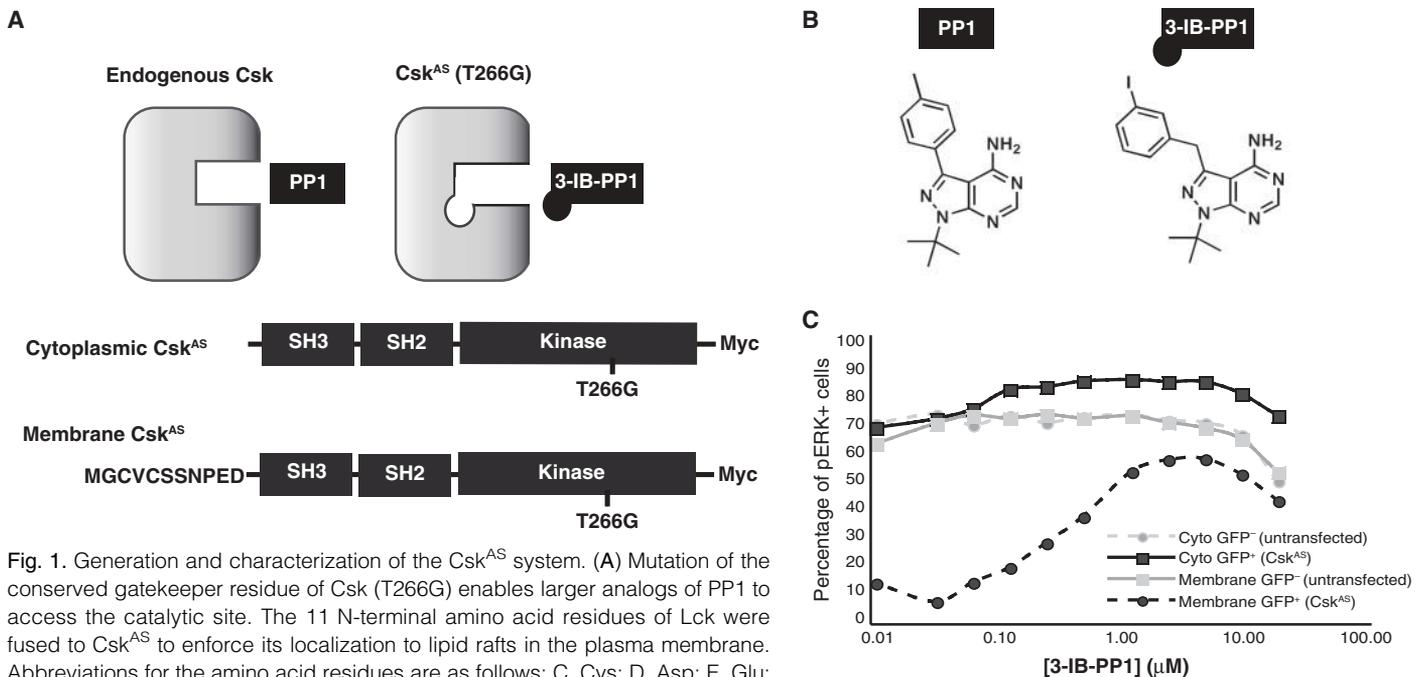
To achieve selective inhibition of Csk, we mutated the “gatekeeper” residue of the catalytic site, thereby enlarging the adenosine triphosphate (ATP)-binding pocket of Csk. The resulting analog-sensitive allele is referred to as *Csk*<sup>AS</sup> (Fig. 1A), and the protein that it encodes can accommodate bulky analogs of the nonselective kinase inhibitor PP1 (13). In addition, we fused the N terminus of Lck to *Csk*<sup>AS</sup> to enrich its localization to lipid rafts, and we refer to this construct as membrane *Csk*<sup>AS</sup>. Because no *Csk*-null T cell lines exist, and because ablation of *Csk* in early T cell development results in aberrant maturation of T cells with altered expression of TCR and co-receptors, we expressed *Csk*<sup>AS</sup> alleles in Jurkat cells, a human T cell line, which has low amounts of *Csk* present at the membrane in resting cells and for which signaling pathways have been well characterized.

Transfection of Jurkat cells with plasmids encoding either membrane-localized wild-type *Csk* (membrane *Csk*<sup>WT</sup>) or membrane *Csk*<sup>AS</sup> efficiently blocked TCR-mediated activation of the cells, as assessed by the change in abundance of the early activation marker CD69 on the cell surface (fig. S1), but was insufficient to block activation in response to phorbol 12-myristate

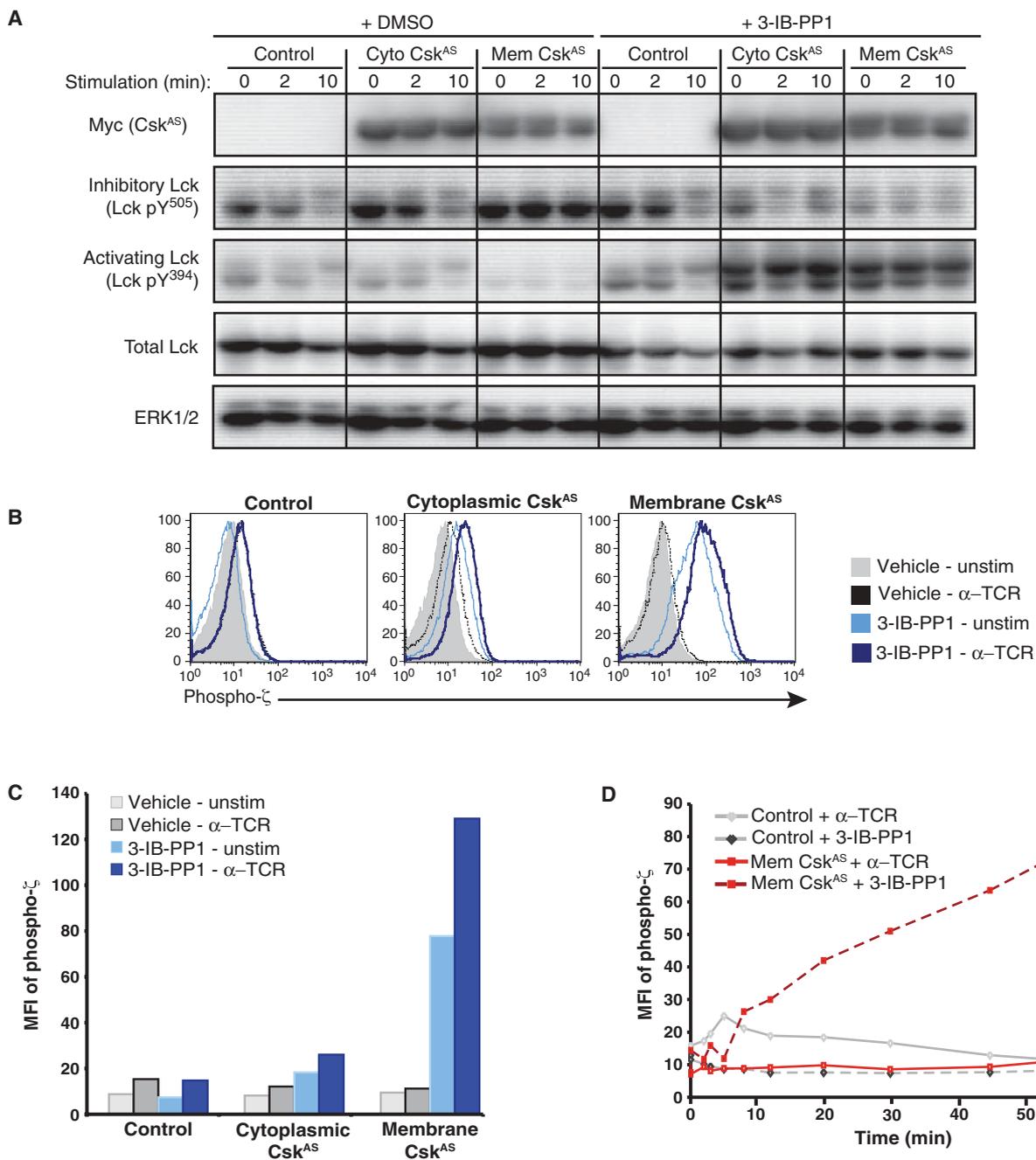
13-acetate (PMA), which activates protein kinase C (PKC) downstream of the TCR. We screened a library of PP1 analogs for their selective ability to block the inhibitory effects of membrane *Csk*<sup>AS</sup> on T cell activation (fig. S1). Whereas the nonselective kinase inhibitor PP1 blocked activation of all cell types, bulky PP1 analogs were unable to block TCR-mediated activation of cells transfected with empty plasmid. We identified 3-IB-PP1 [3-(3-iodobenzyl)-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine] as a selective inhibitor of *Csk*<sup>AS</sup>, but not *Csk*<sup>WT</sup> (Fig. 1B and fig. S1, C and D). Treatment of transfected cells with 3-IB-PP1 blocked the inhibitory function of membrane *Csk*<sup>AS</sup>, as assessed by detection of the phosphorylation of extracellular signal-regulated kinase (ERK), with EC<sub>50</sub> (median effective concentration) values of ~0.5 μM (Fig. 1C); however, when present in similar abundance to that of membrane *Csk*<sup>AS</sup>, cytoplasmic *Csk*<sup>AS</sup> did not inhibit TCR-induced activation of ERK.

### Inhibition of *Csk*<sup>AS</sup> catalytic function activates proximal TCR signaling pathways

To characterize the effects of 3-IB-PP1 on *Csk*<sup>AS</sup> activity, we examined the phosphorylation of the activating (Tyr<sup>394</sup>) and inhibitory (Tyr<sup>505</sup>) tyrosines of Lck after inhibition of *Csk*<sup>AS</sup> with 3-IB-PP1. In control cells, TCR stimulation resulted in decreased phosphorylation of the inhibitory Tyr<sup>505</sup> residue within 2 min, with further decreases seen by 10 min (Fig. 2A). In contrast, total phosphorylation of the activating Tyr<sup>394</sup> residue was not substantially changed in response to TCR stimulation, consistent with a previous study (6); however, one band corresponding to Lck migrated more slowly when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) because of ERK-mediated phosphorylation of Ser<sup>57</sup> in Lck (14). This pattern of Lck phosphorylation was similar in control cells pretreated with 3-IB-PP1, supporting the insensitivity of endogenous *Csk* to 3-IB-PP1. Vehicle-treated cells containing cytoplasmic *Csk*<sup>AS</sup> exhibited a



**Fig. 1.** Generation and characterization of the *Csk*<sup>AS</sup> system. (A) Mutation of the conserved gatekeeper residue of *Csk* (T266G) enables larger analogs of PP1 to access the catalytic site. The 11 N-terminal amino acid residues of Lck were fused to *Csk*<sup>AS</sup> to enforce its localization to lipid rafts in the plasma membrane. Abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; G, Gly; M, Met; N, Asn; S, Ser; and V, Val. (B) Structures of PP1 and 3-IB-PP1. (C) Jurkat cells were transiently cotransfected with plasmid encoding GFP and plasmid encoding either cytoplasmic *Csk*<sup>AS</sup> or membrane *Csk*<sup>AS</sup>. After 16 hours, cells were starved of serum, stimulated with antibody against TCR for 5 min in the presence of DMSO or the indicated doses of 3-IB-PP1, and then analyzed for the presence of pERK. Data show the percentages of live GFP<sup>-</sup> and GFP<sup>+</sup> cells that contain pERK. Data are representative of three independent experiments.



**Fig. 2.** Effect of Csk<sup>AS</sup> on Lck activity. **(A)** Jurkat cells transfected with empty plasmid, plasmid encoding cytoplasmic Csk<sup>AS</sup>, or plasmid encoding membrane Csk<sup>AS</sup> were starved of serum and pretreated with DMSO or 3-IB-PP1 for 25 min, and then lysates were prepared directly or after TCR stimulation for the indicated proteins. Data are representative of three independent experiments. **(B)** Cells cotransfected with plasmid encoding GFP and with empty plasmid, plasmid encoding cytoplasmic Csk<sup>AS</sup>, or plasmid encoding membrane Csk<sup>AS</sup> were starved of serum and then pretreated with DMSO or 3-IB-PP1 (10  $\mu$ M) for 20 min. Cells

were then stimulated with antibody against TCR ( $\alpha$ -TCR) or treated with vehicle (unstim) for 2 min before fixation and flow cytometric analysis for  $\zeta$  chain phosphorylation. Histograms show  $\zeta$  chain phosphorylation in live transfected cells. **(C)** Bar graphs of the data in **(B)** representing the mean fluorescence intensity (MFI) of phospho- $\zeta$  chain in GFP<sup>+</sup> cells. **(D)** Cells cotransfected with plasmid encoding GFP and either empty plasmid or plasmid encoding membrane Csk<sup>AS</sup> were starved of serum and were fixed directly or after stimulation with antibody against the TCR or after treatment with 3-IB-PP1. Data represent the MFI of phospho- $\zeta$  chain in GFP<sup>+</sup> cells and are representative of at least three independent experiments.

moderate increase in the phosphorylation of Lck Tyr<sup>505</sup> in the basal state compared to that in control cells, but they lost Tyr<sup>505</sup> phosphorylation and showed increased phosphorylated Lck Ser<sup>57</sup> in response to TCR stimulation (Fig. 2A). These data are consistent with the notion that only minimal amounts of Csk<sup>AS</sup> and endogenous Csk are localized to the plasma membrane of resting cells and that both are under normal regulatory control mechanisms. In contrast, resting cells containing membrane Csk<sup>AS</sup> showed increased Lck Tyr<sup>505</sup> phosphorylation and only minimally detectable Lck Tyr<sup>394</sup> phosphorylation, which did not change after TCR stimulation. Thus, as previously described (15), membrane Csk<sup>AS</sup> potently inhibited Lck.

The effects of Csk<sup>AS</sup> inhibition by 3-IB-PP1 on the activation of Lck were unanticipated. Whereas pretreatment of control cells with 3-IB-PP1 did not affect the phosphorylation of Lck, pretreatment of cells containing cytoplasmic Csk<sup>AS</sup> or membrane Csk<sup>AS</sup> resulted in reduced phosphorylation of Lck Tyr<sup>505</sup> and increased phosphorylation of Lck Tyr<sup>394</sup>. The activation of Lck in response to Csk<sup>AS</sup> inhibition occurred in the absence of TCR stimulation. These data indicate that inhibition of the catalytic activity of Csk<sup>AS</sup> was sufficient to result in ligand-independent Lck activation, even when only a small amount of Csk<sup>AS</sup> was present at the membrane, as was the case for cytoplasmic Csk<sup>AS</sup>.

Lck phosphorylates the ITAMs of the TCR complex and initiates TCR-mediated signaling. We found that TCR stimulation of control cells induced phosphorylation of the  $\zeta$  and CD3 $\epsilon$  chains, as did treatment of cells containing membrane Csk<sup>AS</sup> with 3-IB-PP1 (fig. S2). Relative to TCR-stimulated control cells, 3-IB-PP1-treated cells containing Csk<sup>AS</sup> showed enhanced phosphorylation of the  $\zeta$  chain, which correlated with the increased recruitment and phosphorylation of the cytoplasmic tyrosine kinase  $\zeta$  chain-associated protein kinase of 70 kD (ZAP-70) (fig. S2). To quantify the amount of  $\zeta$  chain phosphorylation, we used a flow cytometry-based approach. After TCR stimulation, control cells or cells containing cytoplasmic Csk<sup>AS</sup> exhibited a two- to fourfold increase in  $\zeta$  chain phosphorylation compared to that in unstimulated cells (Fig. 2, B and C). In contrast, TCR stimulation of vehicle-treated cells containing membrane Csk<sup>AS</sup> did not exhibit increased  $\zeta$  chain phosphorylation. Although the phosphorylation of Lck Tyr<sup>394</sup> was increased to a similar extent in 3-IB-PP1-pretreated cells containing either cytoplasmic Csk<sup>AS</sup> or membrane Csk<sup>AS</sup> (Fig. 2A), the extent of  $\zeta$  chain phosphorylation was much greater (5- to 10-fold higher) in the cells containing membrane Csk<sup>AS</sup>. In both cell types,  $\zeta$  chain phosphorylation was further increased by TCR stimulation. Control cells responding to TCR stimulation had peak  $\zeta$  chain phosphorylation at 5 min, which returned to the basal state by 45 min (Fig. 2D). In contrast, cells containing membrane Csk<sup>AS</sup> continued to exhibit increased  $\zeta$  chain phosphorylation over the course of 1 hour. Thus, phosphorylation of proximal signaling proteins was augmented and sustained in response to inhibition of Csk<sup>AS</sup>, relative to that in TCR-stimulated control cells. This suggests that rather than returning to basal conditions, enhanced activation of Lck activity occurs when the function of Csk<sup>AS</sup> is inhibited. This effect was amplified in cells that contained the more inhibitory form of Csk<sup>AS</sup> that is targeted to the membrane.

### TCR signaling is augmented and prolonged after inhibition of Csk<sup>AS</sup> activity

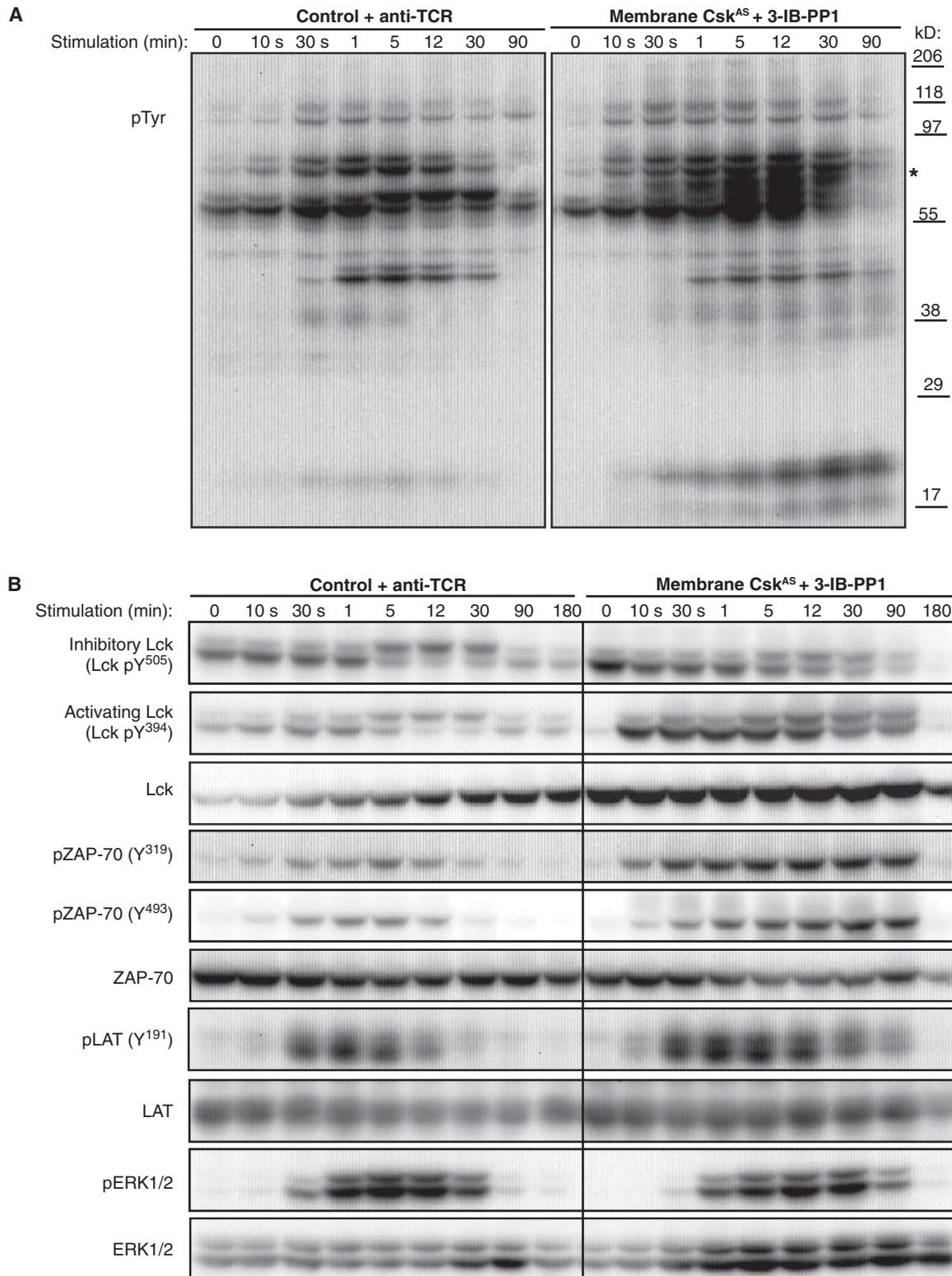
TCR stimulation results in the sudden dephosphorylation of PAG in T cells, causing the rapid dissociation of Csk from the plasma membrane and its movement to the cytoplasm. Csk reappears in membrane lipid rafts within 5 to 10 min of TCR stimulation (9, 16). The increased and sustained  $\zeta$  chain phosphorylation that occurred after inhibition of membrane Csk<sup>AS</sup> suggested that normal mechanisms to control TCR signaling were altered. After treatment with 3-IB-PP1, cells containing membrane Csk<sup>AS</sup> exhib-

ited rapid and prolonged tyrosine phosphorylation of several proteins, including the 21- to 23-kD  $\zeta$  chain (Fig. 3A). The overall pattern of tyrosine phosphorylation was similar to that observed in TCR-stimulated control cells, with the exception of a 65-kD phosphorylated protein seen in the 3-IB-PP1-stimulated cells (Fig. 3A, marked with an asterisk). This suggests that the inhibition of membrane Csk<sup>AS</sup> activates signaling events similar to those driven by TCR stimulation of control cells.

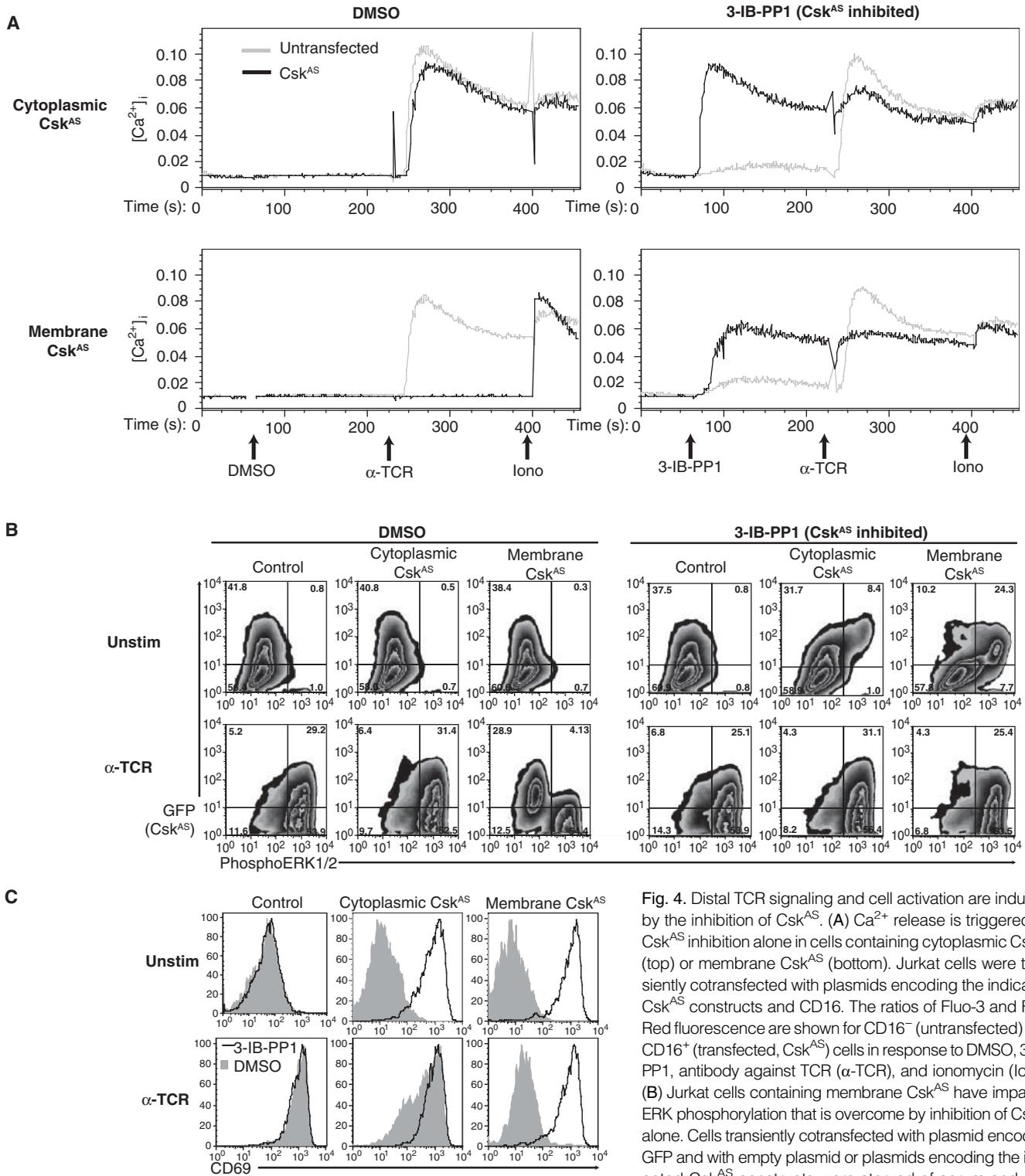
We next compared the phosphorylation states of specific signaling proteins in control cells stimulated through the TCR with those in cells expressing membrane Csk<sup>AS</sup> treated with 3-IB-PP1 (Fig. 3B). The kinase ZAP-70 binds to the Lck-phosphorylated  $\zeta$  chains and is phosphorylated by Lck on Tyr<sup>315</sup> and Tyr<sup>319</sup> to maintain an active conformation (17). Tyr<sup>493</sup> is located in the activation loop of ZAP-70, and it is phosphorylated by either Lck or ZAP-70. We found that both Tyr<sup>319</sup> and Tyr<sup>493</sup> exhibited enhanced and sustained phosphorylation after the inhibition of membrane Csk<sup>AS</sup> (Fig. 3B). Active ZAP-70 is necessary for the phosphorylation of two critical adaptor proteins: linker of activated T cells (LAT) and SH2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76). Together, phosphorylated LAT and SLP-76 create a platform for the recruitment of numerous other molecules necessary for the signaling events downstream of the TCR that direct transcriptional responses and induce cell proliferation. Similar to ZAP-70, enhanced and more prolonged LAT phosphorylation was observed in response to 3-IB-PP1-treated membrane Csk<sup>AS</sup> cells than was seen in control cells stimulated through the TCR, which correlated with sustained signaling downstream of the TCR, including the phosphorylation of ERK. Thus, inhibition of membrane Csk<sup>AS</sup> was sufficient to rapidly activate and induce sustained signaling events that are associated with the canonical TCR signaling pathway, but in a ligand-independent manner.

### Activation of distal signaling events and cellular activation are induced by inhibition of Csk<sup>AS</sup>

Lck-mediated phosphorylation of TCR ITAMs and ZAP-70 is necessary to stimulate increases in the concentration of intracellular calcium ions ( $[Ca^{2+}]_i$ ) and the activation of mitogen-activated protein kinases (MAPKs), which coordinate transcriptional responses, including expression of the genes encoding CD69 and interleukin-2 (IL-2). To examine whether the phosphorylation events described earlier were sufficient to activate downstream signaling and induce cellular activation, we examined  $Ca^{2+}$  flux, phosphorylated ERK (pERK) abundance, and increases in CD69 surface expression. Similar to control cells, cells containing cytoplasmic Csk<sup>AS</sup> exhibited increased  $[Ca^{2+}]_i$  in response to TCR stimulation or ionomycin (Fig. 4A), whereas cells containing membrane Csk<sup>AS</sup> did not exhibit increased  $[Ca^{2+}]_i$  in response to TCR stimulation, consistent with their inability to activate Lck or phosphorylate ITAMs. However, cells containing either cytoplasmic Csk<sup>AS</sup> or membrane Csk<sup>AS</sup> exhibited increased  $[Ca^{2+}]_i$  in response to Csk<sup>AS</sup> inhibition alone, independently of engagement of the TCR. Similarly, vehicle-treated control cells and cells containing cytoplasmic Csk<sup>AS</sup>, but not membrane Csk<sup>AS</sup>, showed increased pERK abundance in response to TCR stimulation (Fig. 4B). A small portion of cells that contained a large amount of cytoplasmic Csk<sup>AS</sup> exhibited increased pERK abundance in response to Csk<sup>AS</sup> inhibition; however, most cells containing membrane Csk<sup>AS</sup> exhibited pERK in response to 3-IB-PP1. Only those cells that contained the greatest amounts of membrane Csk<sup>AS</sup> were unable to exhibit ERK phosphorylation or  $Ca^{2+}$  flux (as was evident by a moderate reduction in  $Ca^{2+}$  flux) upon treatment with 3-IB-PP1. Consistent with a role for sustained Ras-MAPK signaling in driving expression of the gene encoding CD69, cells containing membrane Csk<sup>AS</sup> that were treated with 3-IB-PP1 alone, but not vehicle, nor cells stimulated through the TCR alone, showed an increased abundance of CD69 (Fig. 4C). Cells that contained



**Fig. 3.** Sustained signaling in the context of Csk<sup>AS</sup> inhibition. **(A and B)** Jurkat cells transfected with empty plasmid or plasmid encoding membrane Csk<sup>AS</sup> were starved of serum and then were lysed directly or after treatment for the indicated times with antibody against TCR (left, anti-TCR) or 3-IB-PP1 (right), respectively. **(A)** Total tyrosine phosphorylation and **(B)** phosphorylation of Lck, ZAP-70, LAT, and ERK were assessed by Western blotting analysis with the appropriate antibodies. Data are representative of at least three independent experiments.



**Fig. 4.** Distal TCR signaling and cell activation are induced by the inhibition of Csk<sup>AS</sup>. **(A)** Ca<sup>2+</sup> release is triggered by Csk<sup>AS</sup> inhibition alone in cells containing cytoplasmic Csk<sup>AS</sup> (top) or membrane Csk<sup>AS</sup> (bottom). Jurkat cells were transiently cotransfected with plasmids encoding the indicated Csk<sup>AS</sup> constructs and CD16. The ratios of Fluo-3 and Fura Red fluorescence are shown for CD16<sup>-</sup> (untransfected) and CD16<sup>+</sup> (transfected, Csk<sup>AS</sup>) cells in response to DMSO, 3-IB-PP1, antibody against TCR (α-TCR), and ionomycin (Iono). **(B)** Jurkat cells containing membrane Csk<sup>AS</sup> have impaired ERK phosphorylation that is overcome by inhibition of Csk<sup>AS</sup> alone. Cells transiently cotransfected with plasmid encoding GFP and with empty plasmid or plasmids encoding the indicated Csk<sup>AS</sup> constructs were starved of serum and pre-treated with DMSO or 3-IB-PP1 for 15 min. Cells were then harvested directly (Unstim) or after 2 min of stimulation with an antibody against TCR. Plots show total live cells, with GFP<sup>-</sup> (untransfected) cells in the bottom quadrants and GFP<sup>+</sup> (transfected) cells in the upper quadrants. **(C)** The increase in the abundance of CD69 is impaired in TCR-stimulated cells containing membrane Csk<sup>AS</sup> compared to that in control cells and is induced in response to inhibition of Csk<sup>AS</sup> alone. Transiently transfected Jurkat cells were pretreated with either 3-IB-PP1 or DMSO and were then incubated with antibody against TCR for 18 hours before undergoing flow cytometric analysis for CD69 at the cell surface. Data are representative of two (A) or three (B and C) independent experiments.

treated with DMSO or 3-IB-PP1 for 15 min. Cells were then harvested directly (Unstim) or after 2 min of stimulation with an antibody against TCR. Plots show total live cells, with GFP<sup>-</sup> (untransfected) cells in the bottom quadrants and GFP<sup>+</sup> (transfected) cells in the upper quadrants. **(C)** The increase in the abundance of CD69 is impaired in TCR-stimulated cells containing membrane Csk<sup>AS</sup> compared to that in control cells and is induced in response to inhibition of Csk<sup>AS</sup> alone. Transiently transfected Jurkat cells were pretreated with either 3-IB-PP1 or DMSO and were then incubated with antibody against TCR for 18 hours before undergoing flow cytometric analysis for CD69 at the cell surface. Data are representative of two (A) or three (B and C) independent experiments.

cytoplasmic Csk<sup>AS</sup> induced CD69 expression in response to either inhibition of Csk<sup>AS</sup> or stimulation of the TCR.

We verified that these inducible signaling events in response to inhibition of Csk<sup>AS</sup> also occurred in primary T cells by introducing cytoplasmic Csk<sup>AS</sup> and membrane Csk<sup>AS</sup> into primary mouse T cells. Mouse T cells transfected with empty plasmid alone or with plasmid encoding either Csk<sup>AS</sup> construct exhibited low basal activation of Lck and ERK (fig. S3). TCR stimulation of cells transfected with empty plasmid resulted in a moderate increase in the extent of Lck Tyr<sup>394</sup> phosphorylation and robust ERK activation. Consistent with the data observed in Jurkat cells, inhibition of Csk<sup>AS</sup> with 3-IB-PP1 induced Lck activation and ERK phosphorylation. Thus, we demonstrated that membrane Csk<sup>AS</sup> was a potent inhibitor of TCR stimulation; however, blockade of Csk<sup>AS</sup> catalytic function not only released the Csk-mediated inhibition of Lck, but was also sufficient to activate T cells independently of TCR stimulation. In the presence of 3-IB-PP1, cells containing Csk<sup>AS</sup> showed an enhanced and sustained phosphorylation of signaling proteins relative to the response to TCR stimulation in control cells. These phosphorylation events were most robust when Csk<sup>AS</sup> was targeted to the membrane. Rather than returning to the normal pattern and signaling that requires TCR stimulation, after Csk<sup>AS</sup> inhibition, an enhanced phosphorylation of signaling proteins was observed. Together, these data suggest that ectopic expression of Csk<sup>AS</sup>, particularly when targeted to the membrane, dampens basal signaling in resting cells, which may alter the signaling network as a mechanism to compensate for this impaired basal signaling state. Upon inhibition of Csk<sup>AS</sup>, we hypothesize that this altered basal circuitry is responsible for the unexpected increases in the extent of phosphorylation of signaling proteins. The more prolonged response seen with Csk<sup>AS</sup> inhibition also suggests that Csk might play a role in terminating TCR signaling.

### The enhanced signaling mechanism requires TCR, CD45, and Lck

In resting T cells, the TCR is constitutively internalized and rapidly recycled back to the cell surface. Strong antigenic stimulation induces degradation of the  $\zeta$  chain through Lck-dependent mechanisms. Consistent with the inhibition of basal Lck activity, T cells containing membrane Csk<sup>AS</sup> had decreased basal TCR internalization compared to that in control cells, which resulted in their having enhanced amounts of TCR complexes at the cell surface (fig. S4). After inhibition of Csk<sup>AS</sup>, the amount of TCR at the cell surface was decreased to an abundance similar to that of TCR-stimulated control cells. We found that the presence of the TCR or other ITAM-containing receptors at the cell surface was required for the induction of signaling upon inhibition of Csk<sup>AS</sup>, because no response was observed in TCR $\beta$ -deficient cells transfected with plasmid encoding membrane Csk<sup>AS</sup> and treated with 3-IB-PP1 (fig. S5). However, the increased abundance of the TCR or ITAM-containing receptors was not responsible for the enhanced response observed when Csk<sup>AS</sup> was inhibited, because cell lines that had either low amounts of surface TCR or a fixed amount of a CD8- $\zeta$  chimeric receptor in the absence of the TCR were activated in a manner similar to that of wild-type Jurkat cells (figs. S5 and S6).

Experiments with mutant Jurkat cells deficient in CD45, Lck, ZAP-70, or SLP-76 have revealed the roles of these molecules in the initiation of proximal TCR signaling pathways. We found that signaling downstream of the TCR was blocked in cells deficient in either CD45 (J45 cells) or Lck (J.CaM1 cells) (Fig. 5A). As expected, inhibition of membrane Csk<sup>AS</sup> in these cell lines did not induce downstream signaling events (Fig. 5B). Thus, Lck was required for phosphorylation of the  $\zeta$  chain, and Lck required CD45 for its activation in response to TCR stimulation and activation of signaling in response to Csk<sup>AS</sup> inhibition. ZAP-70-deficient P116 cells were also defective in their ability to respond to TCR stimulation; how-

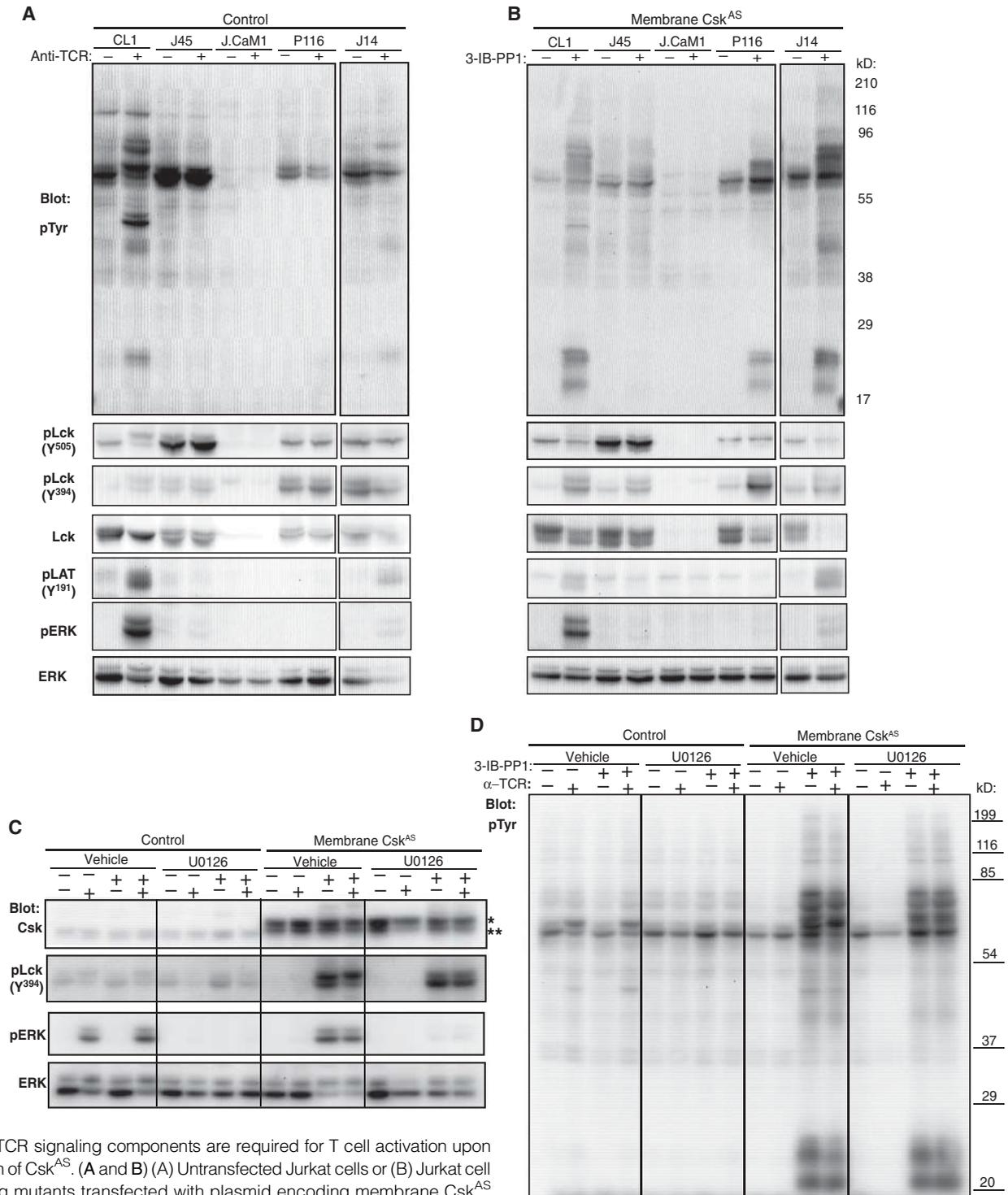
ever, inhibition of membrane Csk<sup>AS</sup> was still sufficient to induce phosphorylation of  $\zeta$  chain and Lck Tyr<sup>394</sup>. The ERK-dependent upper band corresponding to Lck Ser<sup>57</sup> phosphorylation was largely absent after inhibition of Csk<sup>AS</sup>, presumably because of the inability of P116 cells to signal downstream of ZAP-70. SLP-76-deficient J14 cells exhibited activation of proximal signaling events when transfected with either empty plasmid or plasmid encoding membrane Csk<sup>AS</sup> in response to TCR stimulation or 3-IB-PP1 treatment, respectively; however, distal signaling events, such as phosphorylation of ERK, were impaired in both settings. These findings suggest that the mechanism for the phosphorylation of Lck and the  $\zeta$  chain did not require signaling events dependent on ZAP-70 and SLP-76, but required more receptor-proximal components.

Positive and negative feedback loops are implicated in the regulation of Lck to explain signaling differences related to the strength of TCR engagement (14). In one model, strong TCR ligands activate a positive feedback loop that involves ERK-mediated phosphorylation of Lck Ser<sup>57</sup>, which prevents recruitment of the inhibitory SH2 domain-containing tyrosine phosphatase 1 (SHP-1) to Lck to inactivate TCR signaling. Given the importance of Lck activation for the sustained enhanced activation of TCR-dependent signaling in response to Csk<sup>AS</sup> inhibition, we examined the requirement for ERK-mediated phosphorylation of Lck in 3-IB-PP1-induced activation. As predicted, inhibiting the activation of mitogen-activated and extracellular signal-regulated kinase kinase (MEK) with U0126 before treatment with 3-IB-PP1 led to a block in ERK activation after Csk<sup>AS</sup> inhibition (Fig. 5C). In addition, the upper band corresponding to Ser<sup>57</sup>-phosphorylated Lck was reduced in abundance in U0126-pretreated cells compared to that in untreated cells. However, the extent of TCR-proximal signaling (that is, phosphorylation of Lck Tyr<sup>394</sup> and the  $\zeta$  chain) was unaffected by MEK inhibition (Fig. 5, C and D), suggesting that the previously reported ERK-mediated phosphorylation feedback mechanism was not required for enhanced signaling as a result of Csk<sup>AS</sup> inhibition. Thus, we conclude that the enhanced signaling mechanism that occurs in response to Csk<sup>AS</sup> requires the TCR, CD45, and Lck and involves molecules upstream of ZAP-70, SLP-76, and ERK.

### Dok-1 is phosphorylated and interacts with Csk after inhibition of Csk<sup>AS</sup>

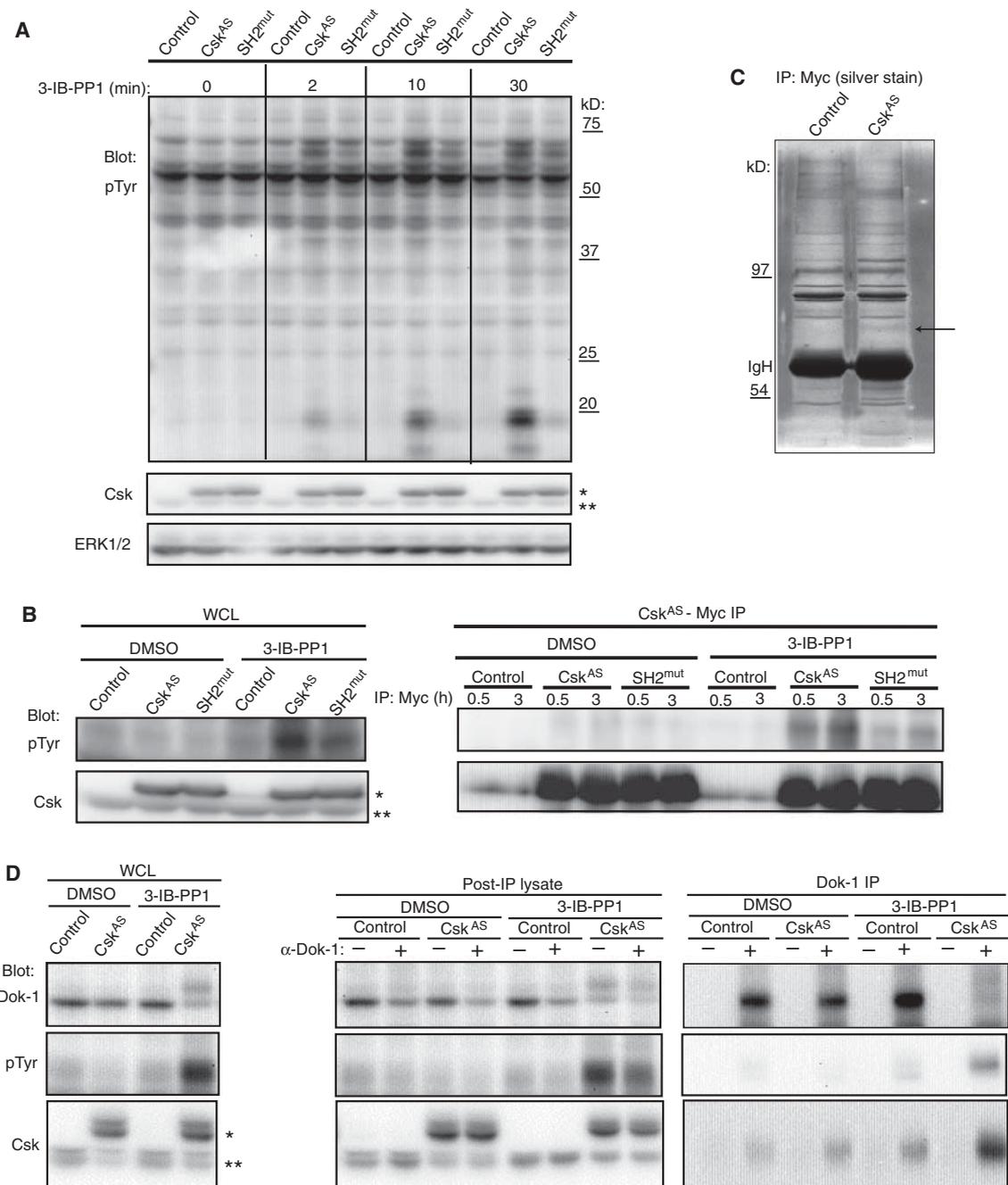
Endogenous Csk is dynamically recruited to the cell membrane because of interactions between its SH2 domain and phosphorylated tyrosines in membrane-anchored adaptor molecules; however, membrane Csk<sup>AS</sup> is constitutively enriched in lipid rafts of the plasma membrane. Despite this, the SH2 domain may still interact with phosphorylated proteins that are important for the regulation of signaling. We postulated that if this were true, then a variant membrane Csk<sup>AS</sup> with a mutation in its SH2 domain would alter signaling differently after treatment with 3-IB-PP1. Indeed, mutation of the SH2 domain of Csk<sup>AS</sup> resulted in a clear diminution in the extent of  $\zeta$  chain phosphorylation as well as that of other proteins after Csk<sup>AS</sup> inhibition (Fig. 6A).

We further observed a tyrosine-phosphorylated band of ~65 kD that was less phosphorylated in cells containing the variant of Csk<sup>AS</sup> with the SH2 mutation than in cells containing Csk<sup>AS</sup>. We were particularly interested in identifying this 65-kD protein because it was uniquely phosphorylated in cells in which either cytoplasmic Csk<sup>AS</sup> or membrane Csk<sup>AS</sup> had been inhibited by 3-IB-PP1 (Figs. 3A and 5B). Phosphorylation of this band occurred within 30 s of Csk<sup>AS</sup> inhibition and was detectable for at least 30 min; however, this 65-kD phosphorylated protein was undetectable before inhibition of Csk or in response to TCR stimulation in control cells. Furthermore, immunoprecipitates of membrane Csk<sup>AS</sup> showed a strong interaction with a tyrosine-phosphorylated protein of 65 kD after inhibition of Csk<sup>AS</sup> by 3-IB-PP1, which was reduced when the SH2 domain of



**Fig. 5.** TCR signaling components are required for T cell activation upon inhibition of Csk<sup>AS</sup>. **(A and B)** (A) Untransfected Jurkat cells or (B) Jurkat cell signaling mutants transfected with plasmid encoding membrane Csk<sup>AS</sup> were purified, starved of serum, and treated with antibody against TCR (anti-TCR) or with 3-IB-PP1 for 10 min and then lysed. Total tyrosine phosphorylation and phosphorylation of Lck, LAT, and ERK were assessed by Western blotting. **(C and D)** Jurkat cells containing membrane Csk<sup>AS</sup> were treated for 2 hours with either vehicle or the MEK inhibitor U0126. Cells were then starved of serum for 30 min in the presence of vehicle or U0126 and then treated with antibody against the TCR or with 3-IB-PP1 for 5 min, as indicated. Cell lysates were

analyzed by Western blotting for total tyrosine phosphorylation, total Csk, and phosphorylation of Lck and ERK. The antibody against Csk recognizes both endogenous Csk (\*\*\*) and Csk<sup>AS</sup> (\*), which has a larger molecular mass than that of endogenous Csk because of the Myc tag, linker sequences, and the membrane-targeting motif. Data are representative of at least three independent experiments.



**Fig. 6.** Dok-1 is uniquely phosphorylated upon Csk<sup>AS</sup> inhibition and interacts with Csk. **(A)** Jurkat cells transfected with empty plasmid, plasmid encoding membrane Csk<sup>AS</sup>, or plasmid encoding the SH2 domain R107K mutant (SH2<sup>mut</sup>) of membrane Csk<sup>AS</sup> were starved of serum and then lysed directly or after treatment with 3-IB-PP1 for the indicated times. Total tyrosine phosphorylation and the amounts of Csk and ERK were assessed by Western blotting analysis. **(B)** Immunoprecipitation (IP) of WT and SH2<sup>mut</sup> membrane Csk<sup>AS</sup> demonstrates that p65 interacts with Csk in an SH2-dependent manner. Cells transfected with empty plasmid or plasmid encoding WT or the SH2 domain mutant of membrane Csk<sup>AS</sup> cells were treated with 3-IB-PP1 or DMSO for 10 min. Lysates were subjected to immunoprecipitation for the indicated times with antibody against Myc and analyzed by Western blotting for the presence of

pTyr and Csk. **(C)** Silver staining of samples immunoprecipitated from control cells or cells containing membrane Csk<sup>AS</sup>. Dok-1 is denoted with an arrow. **(D)** Immunoprecipitation of control cells or cells containing membrane Csk<sup>AS</sup> with antibody against Dok-1. Cells were treated with 3-IB-PP1 or DMSO for 10 min. Lysates were then subjected to immunoprecipitation with antibody against Dok-1 and analyzed by Western blotting for the presence of Dok-1, pTyr, and Csk. Whole-cell lysates (WCL) and lysates after immunoprecipitation were analyzed by Western blotting with an antibody against Csk that recognizes WT (\*\*) and membrane Csk<sup>AS</sup> (\*), whereas the samples immunoprecipitated with the antibody against Dok-1 were analyzed with an antibody against Myc, which recognizes only membrane Csk<sup>AS</sup>. Data are representative of at least two (C) or three (A and B) independent experiments.

Csk<sup>AS</sup> was mutated (Fig. 6B). We hypothesized that the 65-kD protein might interact with Csk<sup>AS</sup> through its SH2 domain and might play a role in the enhanced signaling response. Mass spectrometric analysis of the 65-kD band that coimmunoprecipitated with Myc-tagged Csk<sup>AS</sup> identified various peptides, but only a single peptide that was from the adaptor molecule p62<sup>dok1</sup> (Dok-1) (Fig. 6C and fig. S7). The Dok-1 peptide that we identified was tyrosine-phosphorylated and encompassed Tyr<sup>449</sup>, a known binding site for the SH2 domain of Csk.

Dok-1 is a scaffolding protein that has an inhibitory role in receptor signaling and cell activation in several cell types, including T cells (18). The inhibitory effect of Dok-1 requires its localization to the membrane through the binding of its pleckstrin homology (PH) domain to phospholipids (19). Phosphorylation of Dok-1 on Tyr<sup>449</sup> by SFKs creates the Csk-binding site (20), which was identified by mass spectrometry (MS), and is a likely mechanism for the membrane recruitment of Csk after TCR stimulation. Indeed, we found that Dok-1 phosphorylation was increased when Csk<sup>AS</sup> was inhibited (Fig. 6D). Immunoprecipitation and partial depletion of Dok-1 from cells containing membrane Csk<sup>AS</sup> further confirmed its identity as at least a component of the 65-kD protein band that we observed in lysates. Inhibition of Csk<sup>AS</sup> and phosphorylation of Dok-1 induced an interaction between the two proteins. We suggest that Dok-1 is phosphorylated by Lck and may serve as a regulator that normally senses Lck activity and interacts with Csk at the plasma membrane, particularly after TCR stimulation. The enhanced phosphorylation of Dok-1 by Lck and its association with Csk<sup>AS</sup> may play a role in the enhanced signaling mechanism that we have observed as a result of inhibition of membrane Csk<sup>AS</sup>.

## DISCUSSION

We have generated an analog-sensitive variant of Csk, whose catalytic function can be rapidly and dynamically controlled, which we used to investigate the role of Csk in controlling basal and ligand-induced signaling downstream of the TCR. Because Csk is a ubiquitous regulator of SFK activity, these findings may be applicable to other receptor systems that are dependent on SFK activity. In our studies, constitutive association of Csk<sup>AS</sup> with the plasma membrane potentially inhibited SFK activity and TCR signaling, whereas a cytoplasmic Csk<sup>AS</sup> had no such effect, consistent with previous data (15). Inhibition of either form of Csk<sup>AS</sup> led to the unanticipated activation of the canonical TCR signaling pathway in the complete absence of ligand binding to the receptor. Upon inhibition of membrane Csk<sup>AS</sup>, the enhanced phosphorylation of proximal signaling molecules indicated that ectopic expression of Csk<sup>AS</sup> may have reset the basal signaling tone in the cell. Furthermore, signaling induced by inhibition of Csk<sup>AS</sup> was more sustained than was signaling as a result of direct stimulation of the TCR. These findings suggest the possible involvement of a compensatory adaptive mechanism that monitors and controls basal signaling, at least by SFKs.

These studies suggest that an important function of basal signaling is to monitor and respond to changes in the tonic activity of key signaling components. Direct evidence for basal signaling in T cells has been limited, but some studies suggest a role for basal signaling in survival, transcription, and effector function: Inducible deletion of surface TCR results in a shortened half-life of mature T cells (21), ablation of TCR signaling perturbs gene expression profiles and sensitivity to foreign antigens (22, 23), and expression of the tandem SH2 domains of ZAP-70 increases ITAM phosphorylation in the absence of TCR stimulation (24). A substantial amount of the ZAP-70 tandem SH2 domains associate with ITAMs in resting cells, suggesting that ongoing Lck phosphorylation of the  $\zeta$  chain is protected from phosphatases by the binding of ZAP-70 in the basal state.

Our data imply that basal signaling is monitored and regulated by feedback circuitry.

The ability of Csk<sup>AS</sup> inhibition to rapidly activate Lck in the absence of TCR ligands demonstrates the plasticity in the Lck activation state in resting T cells. A previous study focused on the substantial amount of Lck that is phosphorylated on its activation loop in resting T cells (6). Furthermore, a substantial fraction of Lck molecules contained both pTyr<sup>394</sup> and inhibitory phosphorylation on Tyr<sup>505</sup>. On the basis of these results and our own data, we propose that in resting T cells, a dynamic equilibrium is imposed on Lck by its interactions with Csk and CD45. These interactions lead to a continuous turnover of Tyr<sup>394</sup> and Tyr<sup>505</sup> phosphorylation of Lck and its activity in a basal state and enable rapid and efficient phosphorylation of ITAMs by Lck in response to TCR stimulation. We further propose that this dynamic equilibrium of Lck phosphorylation and activity in resting T cells is monitored and adjusted to signaling perturbations during the inactive basal state.

One possible mechanism to prevent increased phosphorylation of ITAMs by Lck in resting cells would be a requirement for ligand-induced structural changes in the TCR to enable an increased amount of signaling above the basal state to occur (25–27). In this model, the cytoplasmic portion of TCR ITAM tyrosines may bind to the plasma membrane in resting cells. Upon TCR engagement, allosteric or other changes cause the ITAM-containing tyrosines to dissociate from the membrane and become phosphorylated, driving an increased amount of signaling. An alternative model has suggested that allosteric changes in CD3 $\epsilon$  result in accessibility to a polyproline region that binds Nck (28); however, this model has been challenged by knock-in of the CD3 $\epsilon$  proline-rich motif (29). Because TCR signaling pathways are activated by inhibiting Csk<sup>AS</sup> activity in the absence of TCR engagement, neither of these models is complete. Our results suggest that either the CD3 $\epsilon$  chains or the  $\zeta$  chains do not associate with the membrane or that the interactions are highly dynamic and transient. We favor a model in which a low amount of basal signaling is continuously occurring, but is held in check by the balanced action of numerous phosphatases and other inhibitory mechanisms.

Our studies suggest the possibility that adaptation or an alteration in cellular circuitry may have occurred in cells containing the membrane Csk<sup>AS</sup> construct because of decreased basal signaling. Alternatively, the effects of this construct could reflect dominant-negative functions of the 3-IB-PP1-inhibited kinase, whose SH2 and SH3 domains could block the function of endogenous Csk. Although such a dominant-negative effect may contribute to the enhanced signaling response, we favor some amount of cellular adaptation in signaling circuitry to the altered basal signaling. First in support of an adaptation or alteration in basal cellular circuitry is the rapid appearance of the 65-kD phosphoprotein, representing Dok-1, that was not observed in the context of TCR signaling. Second, the amplitude and kinetics of signaling were substantially altered and did not simply mimic the loss of Csk activity from the plasma membrane that was observed after TCR stimulation. Third, in preliminary studies, we have been unable to detect an alteration in the distribution of endogenous Csk by the presence of membrane Csk<sup>AS</sup>. Together, these data suggest that the cellular circuitry in cells containing membrane Csk<sup>AS</sup> has been altered by (or adapted to) the change in basal signaling.

The mechanisms that modulate Csk activity during T cell activation are incompletely understood; however, we suggest that Dok proteins, specifically Dok-1, contribute to the regulation of Csk. During T cell activation, removal of Csk from the immune synapse occurs in response to acute dephosphorylation of Csk-recruiting adaptor proteins, such as PAG; however, this release of Csk is transient, and genetic ablation of PAG has no effect on T cell signaling, suggesting that Csk is likely to interact with additional proteins at the membrane. We suggest that one candidate may

be the inhibitory scaffolding molecule Dok-1, because it is rapidly phosphorylated and associates with Csk after 3-IB-PP1-mediated inhibition of Csk<sup>AS</sup>. Dok proteins bind to phospholipids in the plasma membrane and can interact with target proteins through a protein tyrosine-binding domain. Furthermore, several conserved tyrosines can be phosphorylated by Abl and SFKs after SFK activation (20), including the Csk-binding site Tyr<sup>449</sup>. Indeed, we found that a peptide encompassing this site was tyrosine-phosphorylated and associated with Csk<sup>AS</sup> after treatment with 3-IB-PP1. In fibroblasts, the interaction of Dok-1 pTyr<sup>449</sup> with the SH2 domain of Csk is necessary for the recruitment of Csk to the plasma membrane and the control of Src activity (30). Dok-1 also directly abrogates MAPK signaling by recruiting Ras guanosine triphosphatase-activating proteins (GAPs) to the signalosome. In our studies, membrane Csk<sup>AS</sup> may have caused an alteration in the localization of Dok-1 in the basal state and also competed more effectively than endogenous Csk to interact with Dok-1. Thus, endogenous Csk may not have been able to effectively inhibit or regulate Lck function, which would explain the enhanced signaling seen as a result of 3-IB-PP1-mediated inhibition of Csk<sup>AS</sup> function.

T cells contain Dok-1 and the related family member Dok-2, which together play a critical role in establishing negative feedback loops in TCR signaling. TCR-stimulated T cells deficient in both Dok-1 and Dok-2 exhibit enhanced proliferation and increased cytokine secretion and have prolonged and enhanced phosphorylation of various signaling molecules, including the  $\zeta$  chain, ZAP-70, LAT, and ERK (12, 31), which is reminiscent of our findings in the context of Csk<sup>AS</sup> inhibition. Our attempts to alter the abundance or function of Dok-1 yielded inconsistent results, suggesting that Dok-2 may functionally compensate for Dok-1, as has been previously suggested (12, 32). Future studies will need to clarify the role of Dok proteins as part of a proximal TCR feedback loop to regulate proximal signaling through the recruitment of Csk to the plasma membrane. Moreover, together with Csk, Dok-1 may play an important role in regulating the intensity and termination of the TCR response.

In conclusion, we have used an analog-sensitive variant of Csk to demonstrate that dynamic control of SFK activity downstream of the TCR is necessary for setting basal signal tone and preventing aberrant cellular activation. Because Csk-mediated regulation of SFKs is ubiquitous, these results may be broadly relevant toward understanding how other SFK-associated pathways are regulated in other cell types. Introduction of the Csk<sup>AS</sup> allele into mice will further enable an examination of the regulatory function of Csk in controlling basal signaling tone, and of regulation of TCR-stimulated signaling, in primary cells and during lymphocyte development in the context of endogenous Csk-null T cells. Moreover, the Csk<sup>AS</sup> allele holds potential for being able to exogenously control T cell activity independently of ligand binding.

## MATERIALS AND METHODS

### Cell lines, transfections, and stimulations

The Jurkat T cell line E6-1 was cultured and transfected as described before (33). J.CaM1, J45, P116, and J14 T cell lines have been previously described (34–38). Plasmids encoding green fluorescent protein (GFP) or human CD16 were used as cotransfection controls. Before stimulation, cells were starved of serum at 37°C for at least 25 min. Stimulations were performed in serum-free RPMI at 37°C with a 1:1000 final dilution of C305, an antibody against the V $\beta$ 8 chain of the TCR. 3-IB-PP1 was used at 10  $\mu$ M unless otherwise noted. Before cell lysate preparation, transfected CD16<sup>+</sup> cells were purified with Miltenyi human CD16 microbeads. Cells were treated with the MEK inhibitor U0126 (20  $\mu$ M) for 45 to 120 min.

### Western blotting and flow cytometric analyses

To analyze the phosphorylation of ERK and the  $\zeta$  chain by flow cytometry, we serum-starved and stimulated Jurkat cells as described earlier. The addition of an equivalent volume of Cytofix (BD Pharmingen) was used to stop the stimulations. Cells were collected by centrifugation, washed in fluorescence-activated cell sorting (FACS) buffer, and then resuspended in ice-cold 90% methanol. Cells were incubated on ice for 30 min after which they were washed three times in FACS buffer. Cells were incubated with antibody against phosphorylated p44/42 MAPK (pERK1/2), followed by allophycocyanin (APC)- or phycoerythrin (PE)-conjugated donkey antibody against rabbit immunoglobulin (Ig) (Jackson) or with antibody against CD3 $\zeta$ -pY<sup>142</sup> used at a 1:5 dilution. For the analysis of Ca<sup>2+</sup> flux, cells were loaded with the Ca<sup>2+</sup>-sensitive dyes Fura Red and Fluo-3 (Invitrogen) for 30 min at 37°C in RPMI and 5% fetal bovine serum (FBS), washed, and incubated on ice with Alexa Fluor 647-conjugated antibody against CD16 (BD Pharmingen) to detect transfected cells. Cells were then resuspended in RPMI and warmed to 37°C for 5 min before stimulation. Basal Ca<sup>2+</sup> concentrations were measured for 1 min before the addition of vehicle or 3-IB-PP1 (10  $\mu$ M) for 3 min. Cells were then stimulated through the TCR for 3 min and treated with ionomycin (1  $\mu$ M) as a positive control for 1 min. Ca<sup>2+</sup> increase was measured as the ratio of Fluo-3 to Fura Red fluorescence and was displayed as a function of time for CD16<sup>+</sup> (transfected) cells and CD16<sup>-</sup> (untransfected) cells.

### Antibodies

Antibodies used in this study were against the following targets: LAT-pY<sup>191</sup> (Invitrogen/BioSource); ZAP-70-pY<sup>319</sup> (39), ZAP-70-pY<sup>493</sup>, Src pY<sup>416</sup>, p44/42 MAPK pThr<sup>202</sup>/Tyr<sup>204</sup>, Myc 9B11 (Cell Signaling); Lck (1F6 from J. B. Bolen); pY (4G10, Upstate Biotechnology); LAT, Dok-1 (Abcam); ERK1/2 (Santa Cruz Biotechnology); CD3 $\epsilon$  (clone UCHT.1), CD69 (BD Pharmingen); human CD16, Lck pY<sup>505</sup>, and CD3 $\zeta$ -pY<sup>142</sup> (BD Pharmingen). Horseradish peroxidase-conjugated goat antibody against rabbit IgG (H+L) and mouse IgG (H+L) (Southern Biotech) were used as secondary antibodies. The following antibodies have been described previously: 2F3.2 (ZAP-70) (40) and 6B10.2 (CD3 $\zeta$ ) (39).

### Generation of the Csk<sup>AS</sup> allele

The Csk<sup>AS</sup> allele was generated by cloning mouse Csk from C57BL/6 splenocyte complementary DNA (cDNA) with SuperScript II (Invitrogen) and subcloning it into the plasmid pCR2.1-TOPO (Invitrogen). The sequences encoding the 11 N-terminal amino acid residues of Lck (MGCVCSSNPED) and the Myc tag were added onto the cDNA for the membrane Csk<sup>AS</sup> allele through flexible linker regions. We used the QuikChange kit (Stratagene) to generate the Thr<sup>266</sup>→Gly (T266G) mutation of the gatekeeper residue of Csk. Correctly targeted Csk<sup>T266G</sup> mutations were verified by sequencing and ligated into pEF6/myc-His A (Invitrogen) by digestion with Bam HI and Eco RV. The final construct can be described as 5'-Bam HI-(Lck<sub>11</sub>-SAGGSAGG)-Csk<sup>AS</sup>-SAGGSAGG-Myc-Eco RV-3', where SAGGSAGG is the amino acid sequence of the flexible linker region. Jurkat cells were transfected with plasmids encoding either cytoplasmic Csk<sup>AS</sup> or membrane Csk<sup>AS</sup>, and the cellular localization of these constructs was confirmed by fluorescence microscopy and Western blotting analysis of membrane and cytoplasmic cellular fractions. Whereas only a small portion of cytoplasmic Csk<sup>AS</sup> was recruited to the membrane, which likely represented interactions with endogenous membrane adaptor proteins, membrane Csk<sup>AS</sup> was found almost exclusively at the plasma cell membrane and enriched in lipid rafts. Primers for generation of the Csk<sup>AS</sup> allele were as follows: Cyto-Csk forward: 5'-ggatccatcatgctgcgcaatacaggccgct-3'; Lck11-Csk forward (external): 5'-ggatccatcatggctgtgctcagctcaaacctgaagatagtgctggtgtagtctggtggttc-3'; Lck11-Csk forward (internal): 5'-agtctgctggtgtagtctgctggtgctggcgaatacaggccgctggtccat-3';

Csk-Myc reverse (external): 5'-gatatctacagatcctctctgatgatgtttttgttcacaccagcactaccagcactcaggtg-3'; Csk-Myc reverse (internal): 5'-accacagcactaccagcactcaggtgcagctctgtgggtttgatg-3'; mutagenesis forward: 5'-ggctctacatcgtcggagagtagatcgccaaggg-3'; and mutagenesis reverse: 5'-cccttgccatgtactctccagatgtagacc-3'.

### Description of 3-IB-PP1

A white powder; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.79 (s, 9H), 4.24 (s, 2H), 4.90 (s, 2H), 7.04 (t, 1H), 7.13 (d, 1H), 7.58 (s, 1H), 7.60 (d, 1H), 8.26 (s, 1H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 29.4, 34.9, 60.3, 95.4, 100.8, 127.8, 131.0, 136.6, 137.5, 140.0, 140.8, 154.8, 154.9, 157.7; high-resolution MS (electron ionization) molecular ion calculated for C<sub>16</sub>H<sub>18</sub>N<sub>5</sub> is 407.0685; found, 407.0705. Stock solutions of 3-IB-PP1 (10 mM) were prepared in dimethyl sulfoxide (DMSO), and aliquots for individual use were stored at -80°C.

### In-gel digestion, peptide separation, MS, and protein identification

Immunoprecipitates from 1 × 10<sup>9</sup> cells transfected with plasmid expressing membrane Csk<sup>AS</sup> were eluted from protein G beads with 0.1 M glycine (pH 2.5) and concentrated to 60 μl by centrifugal filtration (Vivaspin 500, Sartorius). The concentrates were resolved by SDS-PAGE, and protein bands were visualized with SYPRO Ruby stain (Invitrogen) according to the manufacturer's protocol. The 65-kD band of interest was excised and subjected to in-gel trypsin digestion together with that of the appropriate negative control band (41). Modified porcine trypsin (Promega) was used at a final concentration of 12.5 ng/μl. Mixtures of proteolytically generated peptides were analyzed by nano-liquid chromatography (nanoLC) tandem MS (MS/MS) with a 2DLC nanoHPLC System (Eksigent) interfaced with an LTQ XL mass spectrometer (Thermo Fisher Scientific) equipped with an Advance ion source (Michrom Bioresources). An LC Phenomenex Onyx monolithic column (100-μm internal diameter, 15 cm in length) was used for both desalting and reversed-phase peptide separation. A 20-min linear gradient from 2% solvent B to 40% solvent B was run at a flow rate of 750 nl/min with solvent A (2% acetonitrile, 0.1% formic acid) and solvent B (90% acetonitrile, 0.1% formic acid). External calibration of the LTQ XL mass spectrometer was performed in MS/MS mode with fragment ions of angiotensin I as references. Peptide fragment ion spectra were obtained by precursor ion selection, which used an automated routine that consisted of a series of one survey MS scan [mass/charge ratio (*m/z*) 400 to 1700], followed by six MS/MS scans (*m/z* 60 to 1500) where helium served as the trap gas and the collision energy was set to 35. Protein identification was accomplished with the MASCOT 2.2 (Matrix Science) search engine. Mammalia taxonomy was searched within the UniProt database (11/27/2009: 513,877 sequences; 180,750,753 residues; taxonomy, *Homo sapiens*; 20,401 sequences) with the following parameters: precursor ion mass tolerance: 0.8 dalton; fragment mass tolerance: 0.8 dalton; tryptic digestion with three missed cleavages, fixed modifications: *S*-carboxyamidomethyl, variable modifications: deamidation (Asn and Gln); Met sulfoxide; and Pyro-Glu (from N-terminal Gln). In-gel digestion of candidate proteins was performed according to the established protocol.

### Cell lines, primary mouse T cell culture, retroviral transduction, and stimulations

A TCRβ-deficient Jurkat cell line stably reconstituted to express either 10% or 60% of the amount of surface TCR in wild-type cells was previously described (42, 43). TCRβ-deficient cells reconstituted with a chimeric CD8-ζ receptor containing the extracellular and transmembrane portions of the CD8 co-receptor linked to the cytoplasmic domain of the ζ chain were previously described (44). Mouse CD4<sup>+</sup> T cells were purified

with the CD4 T Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. Purified T cells were stimulated on plates coated with antibodies against CD3 and CD28 in the presence of IL-2 (100 U/ml) for 24 to 32 hours before retroviral transduction. Csk<sup>AS</sup> was cloned into pMIGR plasmid upstream of the internal ribosomal entry site (IRES) for GFP coexpression, which was used to infect Phoenix packaging cells to generate supernatants for transduction, as previously described by G. Nolan (45). For transductions, medium was gently aspirated from cells and replaced with 1 ml of viral supernatant containing polybrene (8 μg/ml, Sigma). Cells were centrifuged at 1100g for 2 hours at 30°C. Retroviral supernatants were left on cultured cells overnight. Cell culture medium was supplemented with IL-2 for a period of 12 to 24 hours, during which time ~80 to 90% of cells were GFP<sup>+</sup>. Cells were rested from IL-2 for 12 hours and starved of serum for 30 min before stimulation. Cells were stimulated with antibody against CD3 (2C11, 10 μg/ml) and goat antibody against Armenian hamster Ig (Jackson Immunolabs, 50 μg/ml) as indicated. Lysates were prepared by directly lysing cells in an equivalent volume of SDS-PAGE sample buffer. For immunoprecipitations, cells were stimulated as described earlier and immediately washed in ice-cold phosphate-buffered saline. Cells were resuspended in NP-40 lysis buffer with protease and phosphatase inhibitors, and immunoprecipitations were performed according to Abcam's protocol. Dithiothreitol was added to a final concentration of 1% in all lysates, and samples were boiled before running on SDS-PAGE. Proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) by standard Western blotting techniques, and proteins were visualized with SuperSignal ECL reagent (Pierce Biotechnology) and a Kodak Imaging Station.

### TCR internalization assay

For quantification of TCR internalization, Jurkat cells were starved of serum for 30 min at 37°C and then incubated with an antibody against CD3ε (BD Pharmingen, clone UCHT1) on ice in serum-free RPMI for 30 min. Cells were washed in FACS buffer and warmed up to 37°C for stimulation or treatment with 3-IB-PP1 for the indicated times. TCR internalization was stopped by incubating the cells in ice-cold FACS buffer. Cells were divided into two groups to analyze the amount of total CD3ε and that of internalized CD3ε. Cells in the former group were washed in FACS buffer and fixed immediately, whereas cells in the latter group were washed in FACS buffer and had any surface-bound antibody against CD3ε acid-stripped by resuspension in RPMI + 3% FBS (pH 2.0) for 30 s at room temperature. Cells were washed immediately in ice-cold FACS buffer and fixed in 2% paraformaldehyde before flow cytometric analysis of the mean fluorescence intensity (MFI) of CD3ε. To calculate the amount of internalized TCR, we used the following equation: (Stx - St0)/(Tt0 - St0) × 100, where Stx is the MFI of cells that were acid-stripped after stimulation, St0 is the MFI of cells that were acid-stripped but not stimulated, and Tt0 is the total MFI of cells without stripping. Controls to verify cell viability and other surface markers were used to verify quality of data.

### SUPPLEMENTARY MATERIALS

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Fig. S1. Screen of PP1 analogs for a specific inhibitor of Csk<sup>AS</sup>.

Fig. S2. Phosphorylation of ζ chain and its association with ZAP-70 upon inhibition of Csk<sup>AS</sup>.

Fig. S3. Inhibition of Csk<sup>AS</sup> in primary mouse T cells activates Lck and ERK.

Fig. S4. Csk<sup>AS</sup> activity regulates the amount of the TCR at the cell surface.

Fig. S5. Cellular activation induced by Csk<sup>AS</sup> inhibition requires TCR at the cell surface, but is not dependent on the amount of TCR.

Fig. S6. Increase in TCR abundance in response to Csk<sup>AS</sup> is not required for cellular activation upon inhibition of Csk<sup>AS</sup>.

Fig. S7. Identification of Dok-1 as p65 by mass spectrometric analysis of Csk<sup>AS</sup> immunoprecipitates.

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## Feedback Circuits Monitor and Adjust Basal Lck-Dependent Events in T Cell Receptor Signaling

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### Poised for Activation

The activity of the Src family kinase Lck is blocked by the phosphorylation of an inhibitory tyrosine residue by the cytoplasmic C-terminal Src kinase, Csk. In response to engagement of the T cell receptor (TCR) by agonist peptide, Csk is lost from the plasma membrane, and dephosphorylation of the inhibitory residue by the phosphatase CD45 enables Lck to activate signaling downstream of the TCR. In experiments in T cells containing a membrane-anchored, analog-sensitive mutant of Csk, Schoenborn *et al.* found that inhibition of Csk was sufficient to induce activation of Lck independently of ligand binding to the TCR and that the resulting signaling was enhanced and prolonged compared to that in TCR-stimulated cells. Together, these findings demonstrate ligand-independent, basal TCR signaling, which suggests that molecules downstream of the TCR are poised to respond to receptor activation but are restrained by the inhibitory kinase Csk.

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