Active Ca^{2+}/Calmodulin-Dependent Protein Kinase IIγB Impairs Positive Selection of T Cells by Modulating TCR Signaling[1]

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T cell development is regulated at two critical checkpoints that involve signaling events through the TCR. These signals are propagated by kinases of the Src and Syk families, which activate several adaptor molecules to trigger Ca^{2+} release and, in turn, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activation. In this study, we show that a constitutively active form of CaMKII antagonizes TCR signaling and impairs positive selection of thymocytes in mice. Following TCR engagement, active CaMKII decreases TCR-mediated CD3ζ chain phosphorylation and ZAP70 recruitment, preventing further downstream events. Therefore, we propose that CaMKII belongs to a negative-feedback loop that modulates the strength of the TCR signal through the tyrosine phosphatase Src homology 2 domain-containing phosphatase 2 (SHP-2). ***The Journal of Immunology,*** **2005, 175: 656–664.**

The development of T cells is an ordered process that is tightly regulated by signals generated through the TCR. The pre-TCR governs the transition of immature thymocytes from the double-negative (DN)[3] to the double-positive (DP) stage. During the DP stage, after rearrangement of the TCRα chain is complete, thymocytes must receive a signal of the appropriate strength through the TCR to proceed through development. The absence of a signal results in death by neglect, an intermediate signal promotes positive selection and development into $CD4^+$ or $CD8^+$ single-positive (SP) cells, and a strong signal induces apoptosis and negative selection (1).

Signals through the TCR are propagated by several molecules. Following TCR engagement, ITAMs within the cytoplasmic tails of the CD3 subunits become phosphorylated by LCK and, to a lesser extent, by FYN (2). ZAP70 binds to phosphorylated ITAMs and undergoes full catalytic activation by LCK (3). Two identified substrates of activated ZAP70 are the adapter proteins Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76) and linker for activation of T cells (LAT) (4, 5). Phosphorylated LAT induces the formation of a multimolecular complex including VAV, NCK, GADS, phospholipase C (PLC)-γ, PI3K, CBL, SLAP-130, and GRB2/SOS/RAS (6). The formation of this complex initiates an increase of intracellular Ca^{2+} and inositol phosphates, which ultimately activate members of the MAPK family and transcription factors of the NF-AT and NF-κB families (7).

Among these various signaling molecules, intracellular Ca^{2+} plays an important role in translating the signal through the TCR into a particular cellular outcome (8). For example, high-affinity peptide-TCR interactions induce high-amplitude Ca^{2+} elevations and negative selection, whereas low-affinity peptide-TCR interactions induce low-amplitude Ca^{2+} elevations and positive selection (9). Moreover, TCR stimulation initiates either amplitude- or frequency-encoded Ca^{2+} changes depending on the maturation state of thymocytes, the avidity of TCR interaction, and the coreceptor involved in the engagement (9). Therefore, the transduction of many cellular stimuli during T cell development results in oscillations in the intracellular concentration of Ca^{2+} and influences the fate of thymocytes.

Members of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) family are biochemical decoders of intracellular Ca^{2+} oscillations. CAMKII is encoded by four genes (α, β, γ, and δ) that have distinct expression patterns (10). The γ and δ isoforms are expressed in T cells and are activated by binding with the Ca^{2+}/calmodulin complex after Ca^{2+} influx. Following activation, CaMKII undergoes an important autophosphorylation of Thr^{287} in the inhibitory domain (11). This autophosphorylation disrupts the kinase regulatory domain, thereby converting the enzyme into a Ca^{2+}-independent (autonomous) form (12). The level of autonomous CaMKII activity is dependent on the initial frequency of Ca^{2+} oscillations produced by various stimuli (13), and thus, CaMKII acts as a biochemical Ca^{2+} decoder. Substitution of Thr^{287} by an aspartic acid mimics autophosphorylation and generates a kinase that has up to 80% autonomous activity (14, 15). Previously, we generated transgenic mice expressing the CAMKII-T287D mutation (CaMKIIγB*) in the T cell lineage, and demonstrated that this active form of CAMKIIγB increased the life span of DP thymocytes and the number of mature T cells with a memory phenotype (16). In this study, we provide evidence for another role played by CAMKII. Expression of CaMKIIγB* impaired positive selection due to an antagonistic effect on TCR signaling. Our results suggest that CaMKII plays a previously unrecognized, regulatory, feedback role in TCR signaling of thymocytes by sustaining SH2 domain-containing phosphatase 2 (SHP-2) interaction with the TCR complex.

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[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; LAT, linker for activation of T cells; PLC, phospholipase C; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CaMKIIγB*, Ca^{2+}-independent mutant of CaMKIIγB; SH, Src homology; SHP, SH2 domain-containing phosphatase; WT, wild type; PTPase, phosphatase; PP2A, protein phosphatase 2A; PTPN-2, protein tyrosine phosphatase, nonreceptor type 2; NMDA, *N*-methyl-D-asparate.

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Materials and Methods

Animals

All mice were bred and maintained in the animal facility at the University of California, San Diego, in sterile isolator cages on irradiated food and autoclaved water. AND, H-Y TCR transgenic mice, and CaMKIIγB* transgenic mice (founders 50, 66, and 32) were previously described (16–18). These mice have been backcrossed to C57BL/6J for 11 generations. MHC double-knockout mice were a generous gift from C. Surh (The Scripps Research Institute, La Jolla, CA), and were generated through the mating of $\beta_2M^{-/-}$ and $I\text{-}A^{b-/-}$ deficient mice (19) on C57BL/6J genetic background.

Flow cytometry

Abs to CD4 (CT-CD4) and CD8α (CT-CD8α) were from Caltag. The following Abs from BD Pharmingen were used: TCRβ (H57), CD3ε (145-2C11), CD4 (GK1.5), CD5 (53-7.3), CD24 (M1/69), CD25 (7D.4), CD69 (H1.2F3), and CD44 (IM7). T3.70 Ab was produced in our laboratory and revealed with goat anti-mouse IgG (Caltag).

Western blots

A total of 4×10^7 thymocytes was lysed in 200 μl of 1% Triton X-100 buffer (100 mM NaCl, 50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol) supplemented with protease inhibitor mixture set I and phosphatase (PTPase) inhibitor mixture set II (Calbiochem) at 4°C for 15 min, followed by a 20-min high-speed spin to remove debris. Following electrophoresis, samples were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blocked with PBS-0.1% Tween 20 supplemented with 5% BSA (Sigma-Aldrich). Blots were developed using ECL (Amersham Biosciences) and analyzed on a Versadoc 3000 (Bio-Rad) or a PhosphorImager Storm 960 (Molecular Dynamics). Anti-phospho-src family Tyr^{416} was the anti-Tyr^{394}-LCK Ab (Cell Signaling Technology). Anti-phospho-Tyr (clone 4G10), anti-CD4 (H-370), and anti-CD3ε (M-20) were from Santa Cruz Biotechnology.

Kinase assay

LCK and FYN activity were monitored by an in vitro kinase assay as follows. Thymocytes were homogenized in lysis buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EGTA, 5 mM MgCl2, 0.5 μg/ml leupeptin, 200 μM sodium vanadate, 1% Triton X-100, 10% glycerol), and the lysate was incubated with an anti-LCK or an anti-FYN Ab or a control Ig at 4°C for 90 min. The immunocomplex was precipitated with protein A-Sepharose. Kinase reactions were conducted with the immunoprecipitates at 37°C for 20 min in 50 μl of kinase buffer (20 mM PIPES (pH 7.6), 10 mM $MgCl_2$, 1 mM EGTA) containing 5 μCi of [^{32}P]ATP (Amersham Biosciences) supplemented by an exogenous substrate, enolase (1 μg; Sigma-Aldrich). The reaction was stopped by adding 10 μl of Laemmli sample loading buffer and was analyzed by SDS-PAGE under reducing conditions. To visualize tyrosine phosphorylation, the gel was subjected to film autoradiography. ^{32}P incorporation into enolase (41 kDa) was measured by densitometry using ImageQuant (Amersham Biosciences).

Retrovirus infection

Retrovirus was produced by transfection of 293T cells by using a Ca^{2+} phosphate transfection kit (Invitrogen Life Technologies). A total of 2.5×10^6 T cells was infected by spinning at 32°C for 120 min. After 8 h at 32°C, cells were transferred to 37°C and analyzed 24–48 h later. Thy1.1 or hu-CD25 were used as a cell surface marker of infection. $Thy1.1^+$ or hu-$CD25^+$ cells were positively selected using magnetic beads (Miltenyi Biotec).

Calcium flux

A total of 10^7 thymocytes were labeled in DMEM containing 10 mM HEPES, 2 μM Fura Red (Molecular Probes), and 1 μM Fluo 4 (Molecular Probes), in the presence of 0.2% Pluronic (Molecular Probes) for 25 min at room temperature. Cells were washed in DMEM, 1% FCS with 10 mM HEPES, twice. After adding stimulating Abs (CD3-biotin and/or CD4-biotin), the cells were incubated on ice for 20 min, washed twice, and resuspended in DMEM/1% FCS with 10 mM HEPES. The cells were collected on a FACSCalibur (BD Biosciences) for 30 s to establish a baseline FL1/FL3 ratio (reflective of intracellular free Ca^{2+} levels) for unstimulated cells. Prewarmed streptavidin (4 μg/ml; Pierce) was added to the cells, and the changes in the FL1/FL3 ratio was monitored for the following 6 min. Changes in intracellular Ca^{2+} levels were analyzed by using FlowJo software (Tree Star) (20).

Results

Altered thymocyte populations in CaMKIIγB mice*

Although the human CaMKIIγB and γC isoforms were cloned from T cells (15), the expression of these isoforms in thymocyte subsets was unknown. Western blot analysis indicated that CaMKIIγB and γC are expressed in all thymocyte subsets (Fig. 1*A*). To evaluate the role of CaMKIIγB in T cell development, we generated transgenic mice expressing CaMKIIγB* under the control of the human CD2 promoter or the mouse CD4 promoter (16). Several founders were obtained that expressed the transgene at different levels; founder CaMKIIγB*-50 (F50), founder CaMKIIγB*-66 (F66), and founder CaMKIIγB*-32 (F32) expressed the transgene in the thymus at about five times, four times, and two times the level of endogenous protein, respectively (Fig. 1*B*). In F32, the CD2 promoter drives expression of the transgene, whereas in F50 and F66, the transgenes contain the mouse CD4 promoter.

To test whether expression of active CAMKIIγB altered T cell development, we analyzed the thymocyte cellularity and the developmental subsets from wild-type (WT) and CaMKIIγB* mice. The fraction of DP thymocytes was increased in CaMKIIγB* mice (percentage of DPs in WT, 82.5 ± 3%; F50, 92 ± 1.4%; F66, 85.5 ± 3%; F32, 90 ± 1%), whereas the percentage of SP cells was decreased in CaMKIIγB* mice compared with WT mice (percentage of SPs in WT, 13.4 ± 2.6%; F50, 3 ± 0.6%; F66, 9.9 ± 2.3%; F32, 6.4 ± 1%; Fig. 2*A*). All founders had a decrease in the number of both $CD4^+$ and $CD8^+$ SP thymocytes (Fig. 2*B*). Interestingly, the decrease of SP thymocytes was more severe in the founder with the highest transgene expression level. No major differences were observed in the number of DP and DN thymocytes in mice between 6 and 8 wk of age. In mice older than 12 wk, the number of DP and DN thymocytes was increased up to 2-fold in F32 as described in Bui et al. (16), suggesting that there could be an increase in T cell precursors in mice expressing the transgene

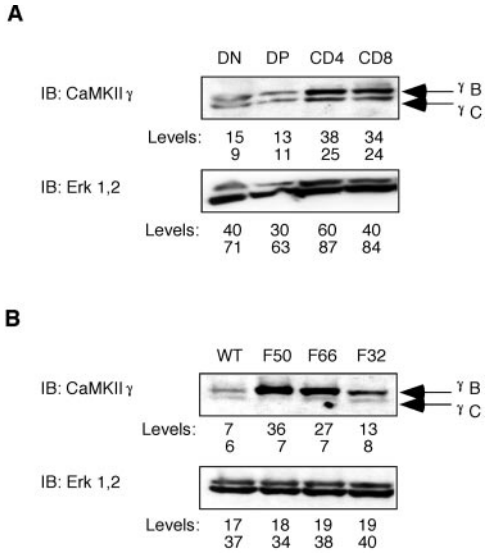

FIGURE 1. CaMKIIγB* is expressed in all thymocyte subsets. *A*, Expression of CaMKIIγ was determined by Western blotting on purified DN, DP, CD4, and CD8 thymocytes. *B*, The level of CaMKIIγB* transgene expression in each founder was analyzed by Western blot on total thymocytes, and the level of Erk expression was used as loading control. The numbers indicate the level of expression obtained by densitometry (Versadoc; Bio-Rad).

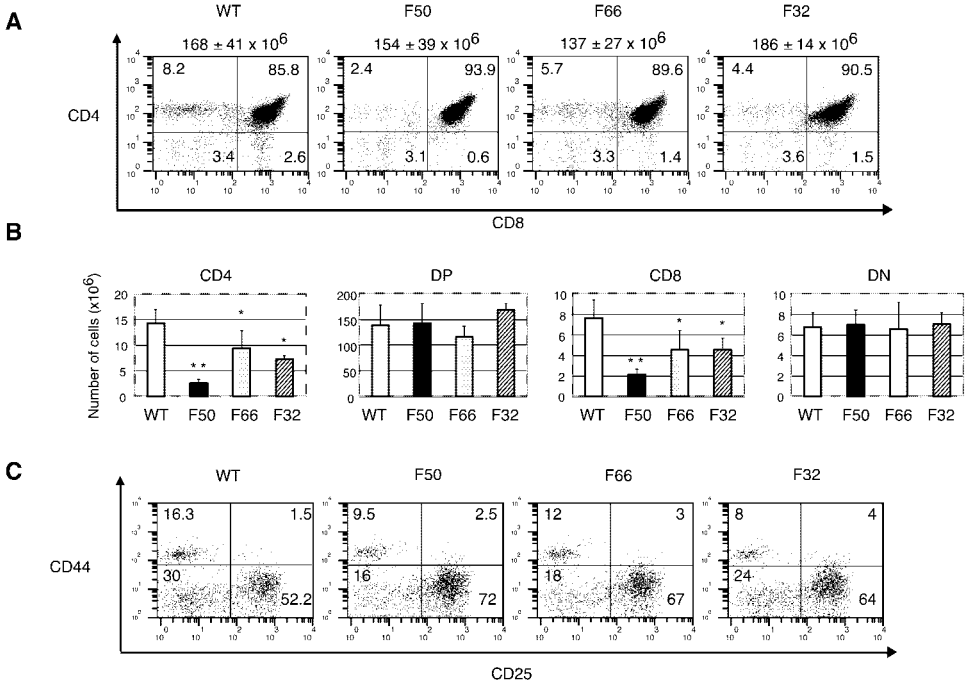

FIGURE 2. T cell development in CaMKIIγB* mice. *A*, Thymocytes were enumerated and analyzed by flow cytometry for CD4 and CD8 expression. The percentage of thymocytes in each subset is indicated. The numbers above the plots are the total number of thymocytes. *B*, Absolute numbers of CD4 SP, DP, CD8 SP, and DN cells found in WT and CaMKIIγB* mice at 6–8 wk of age ($n = 9$ for WT; $n = 9$ for F50; $n = 13$ for F66; $n = 7$ for F32). *, $p < 0.001$; **, $p < 0.0001$ using the two-tailed Student test. *C*, The plots represent the level of CD44 and CD25 on $CD4^-CD8^-IgD^-\gamma\delta^-MHCII^-$ thymocytes, and the percentage of each DN subset is indicated.

under the control of the CD2 promoter (data not shown). Therefore, CaMKIIγB* plays a role in T cell development and alters the selection of SP thymocytes.

The earliest T cell precursors are found among DN thymocytes, which can be further subdivided into four discrete stages defined by the differential expression of the IL-2R α-chain (CD25) and of CD44: $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3), and $CD44^-CD25^-$ (DN4) (21). In CaMKIIγB* mice, the percentage of DN3 thymocytes was increased significantly in all founders (percentage of DN3 in WT, 51 ± 10.2%; F50, 60.3 ± 6.9%; F66, 61.3 ± 7.1%; F32, 61 ± 3.8%; Fig. 2*C*). These data indicate that the transition from the DN3 stage to the DN4 stage was impaired in CaMKIIγB* mice. The assembly of *TCRβ* genes proceeds at the DN3 stage, and upon successful rearrangement and expression, DN3 thymocytes undergo proliferative expansion and differentiate into DP cells (22). Taken together, these data suggest that CaMKIIγB* plays a role in two checkpoints where the quality of the TCR is evaluated: during β selection in the DN stage and positive selection at the DP stage.

Decreased positive selection in CaMKIIγB mice*

To rule out the possibility that the decrease in SP numbers was due to inhibition of TCR rearrangement by CaMKIIγB*, we bred CaMKIIγB* F50 mice with either AND or H-Y TCR transgenic mice. Thymocytes bearing the AND TCR transgene are positively

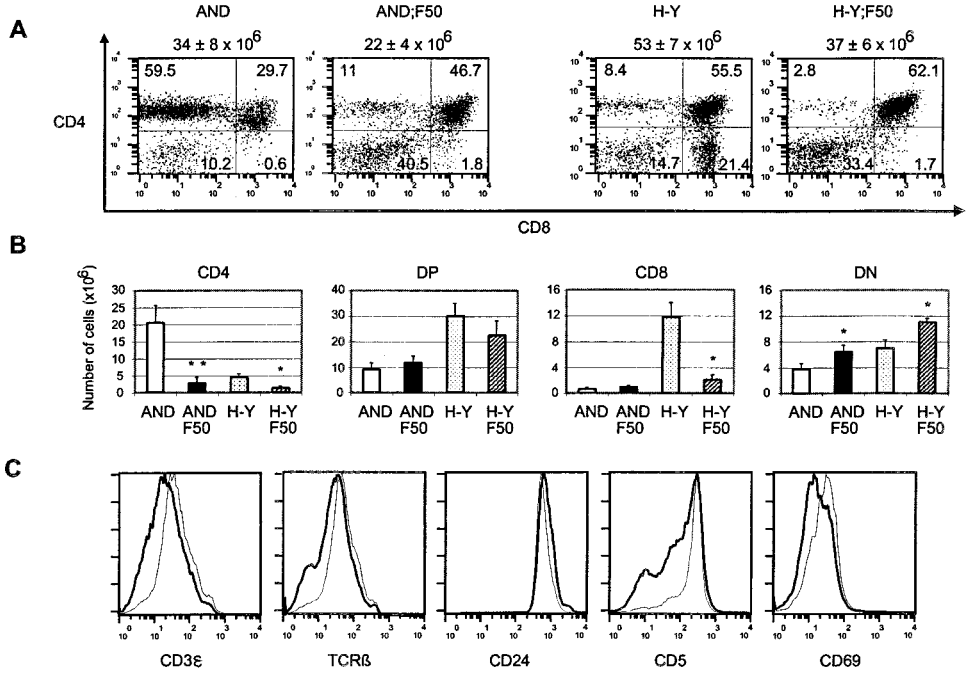

FIGURE 3. Altered T cell development in TCR transgenic CaMKIIγB* mice. *A*, Thymocytes were stained with anti-CD4 and anti-CD8 to show the range of selection. *B*, Absolute numbers of CD4 SP, DP, CD8 SP, and DN cells found in mice at 8 wk of age ($n = 7$ for AND; $n = 7$ for AND;F50; $n = 5$ for H-Y; $n = 6$ for H-Y;F50). *, $p < 0.01$; **, $p < 0.0001$ using the two-tailed Student test. *C*, The level of expression of CD3ε, TCRβ, CD24, CD5, and CD69 on DP thymocytes is shown. The thick line represents AND;F50 thymocytes, and the thin line represents AND thymocytes.

selected by I-A^b MHC class II molecules, resulting in large numbers of CD4$^+$ cells that express the Vα11 TCR chain (18). H-Y TCR transgenic mice express a MHC class I-restricted TCR directed against the male-specific Ag presented by H-2^b MHC class I molecules (17). Female H-Y transgenic mice positively select H-Y TCR$^+$ cells into the CD8 lineage. AND;F50 and H-Y;F50 thymi exhibited dramatic reductions in the percentage and absolute numbers of CD4$^+$ and CD8$^+$ cells, respectively. In addition, the percentages and numbers of DN thymocytes were increased (Fig. 3, *A* and *B*). However, there was no significant difference in the number of DP thymocytes. We also observed a reduced number of CD4 SP in AND;F66 mice (AND CD4 SP, $19.6 \pm 4.8 \times 10^6$ cells; AND;F66 CD4 SP, $12.8 \pm 2.7 \times 10^6$ cells; $n = 7$; $p < 0.01$). Therefore, positive selection was impaired in CaMKIIγB* mice even in the presence of a transgenic TCR. In addition, the expression level of cell surface markers confirmed the defect in positive selection (23, 24). AND;F50 DP thymocytes expressed a lower level of CD3ϵ, TCRβ, CD5, and CD69, and a higher level of CD24 compared with AND DP thymocytes (Fig. 3*C*). Together, these data suggest that CaMKIIγB* negatively regulates T cell differentiation possibly by inhibiting TCR signaling and, to a lower extent, pre-TCR signaling.

Impaired mobilization of intracellular Ca^{2+} in thymocytes from CaMKIIγB mice*

To determine which signaling pathways were affected by the CaMKIIγB* transgene, the release of free Ca^{2+} was measured as a function of time after stimulation of DP thymocytes with anti-CD3ϵ and anti-CD4 Abs. We were concerned that the lower level of CD3ϵ on F50 DP thymocytes could reduce the strength of the stimulation with anti-CD3ϵ Ab. To overcome this issue, we took advantage of MHC$^{-/-}$ mice (19) and the AND TCR, which is not positively or negatively selected on an H-2^d haplotype (25). Therefore, 98% of the thymocytes in AND^d;RAG2$^{-/-}$ (ANDR) and MHC$^{-/-}$ thymi are preselection DP thymocytes. Cell surface staining showed that AND^d;RAG2$^{-/-}$;F50 (ANDR50) DP thymocytes expressed the same level of CD3ϵ, CD24, CD5, CD69, and CD4 as ANDR DP thymocytes (Fig. 4*A*). Similar results were obtained with MHC$^{-/-}$ mice (data not shown). To measure the Ca^{2+} flux, thymocytes were labeled with Fluo-4 and Fura Red, stimulated by cross-linking biotin-conjugated anti-CD3ϵ and anti-CD4 Abs with streptavidin, and the release of Ca^{2+} was measured over time by flow cytometry. The Ca^{2+} flux in response to ionomycin was the same for ANDR and ANDR50 thymocytes, which suggests that the mechanism of Ca^{2+} release within the endoplasmic reticulum stores is normal. However, in response to CD3/CD4 cross-linking, the Ca^{2+} flux elicited in ANDR50 thymocytes was one-third that of ANDR thymocytes (Fig. 4*B*). These results suggest that biochemical events leading to Ca^{2+} release are downmodulated in CaMKIIγB* mice.

CaMKIIγB inhibits TCR-proximal tyrosine phosphorylation*

Ca^{2+} flux that is initiated by TCR signaling occurs as a result of a series of molecular events that include tyrosine phosphorylation of CD3ζ, recruitment of ZAP70, phosphorylation of PLCγ, inositol 1,4,5-triphosphate production, and release of Ca^{2+} stores from the endoplasmic reticulum. To investigate the mechanism by which CaMKIIγB* decreased Ca^{2+} flux, we next looked at PLCγ activation. In MHC$^{-/-}$ thymocytes, PLCγ was phosphorylated after TCR activation; however, the amount of phosphorylated PLCγ was dramatically reduced in MHC$^{-/-}$;F50 thymocytes (Fig. 4*C*). Subsequently, we examined signaling events more proximal to the

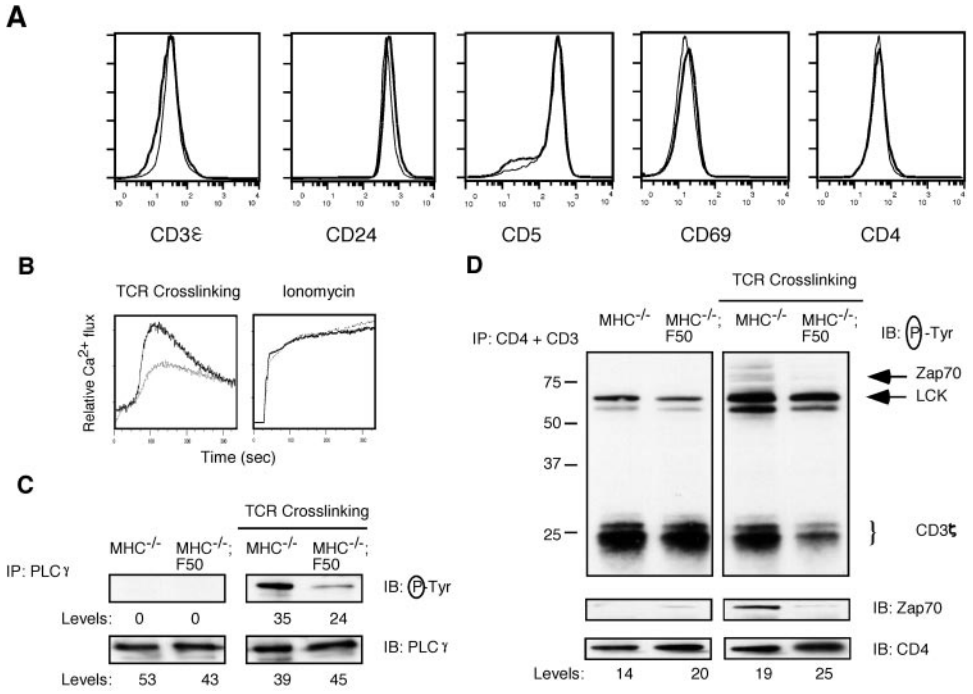

FIGURE 4. Reduced TCR signaling in CaMKIIγB* mice. *A*, Expression of surface markers on DP thymocytes from ANDR mice (thin line) are overlaid on ANDRF50 (thick line) DP thymocytes. *B*, Ca^{2+} mobilization in response to TCR stimulation (*left*) or ionomycin (*right*) is compared in DP thymocytes from ANDR (bold line) and ANDRF50 (dotted line) mice. *C*, Analysis of PLCγ tyrosine phosphorylation. Thymocytes were stimulated for 2 min with anti-CD3ϵ and anti-CD4 Ab and lysed. PLCγ was immunoprecipitated and analyzed by Western blot for anti-phosphotyrosine. The membrane was stripped and reprobed with anti-PLCγ as a loading control. The numbers indicate the level of expression obtained by densitometry (ImageQuant; Amersham Biosciences). *D*, Analysis of CD3ϵ/CD4 associated proteins. CD3ϵ and CD4 were immunoprecipitated, and analyzed by Western blot for anti-phosphotyrosine, anti-ZAP70, and anti-CD4, the latter as a loading control. Molecular masses (in kilodaltons) are indicated.

TCR, and found that the amount of ZAP70 associated with the TCR after cross-linking was significantly reduced in $MHC^{-/-}$;F50 thymocytes compared with $MHC^{-/-}$ thymocytes (Fig. 4*D*). This was evident in the amount of phosphorylated and nonphosphorylated ZAP70 associated with the TCR complex. In addition, $MHC^{-/-}$;F50 thymocytes exhibited decreased CD3ζ phosphorylation after TCR cross-linking (Fig. 4*D*). Finally, there was less Erk phosphorylation after TCR cross-linking in CaMKIIγB* thymocytes, although it was similar in response to PMA/ionomycin (data not shown). Similar results were obtained in ANDR compared with ANDR50 mice (data not shown). There was no difference in the level of expression of LCK, ZAP70, LAT, SLP-76, PLCγ, and CD3ζ in CaMKIIγB* thymocytes (data not shown), which rules out the possibility that CaMKIIγB* regulates the transcription or the translation of any of these signaling molecules. In summary, CD3ζ phosphorylation, ZAP70 activation and association with CD3, and PLCγ activation were all impaired in the presence of constitutively active CaMKIIγB*. This suggests that CaMKIIγB* inhibits ITAM phosphorylation and, in turn, blocks activation of PLCγ and prevents optimal release of intracellular Ca^{2+} stores.

*Impaired mobilization of intracellular Ca^{2+} in a cell line expressing CaMKIIγB**

The simplest interpretation of the data is that CaMKIIγB* antagonizes TCR signaling in DP thymocytes. However, this inhibition could be due to abnormal T cell development because we observed a partial block at the DN3 stage (Fig. 2*C*). Thus, we confirmed our results by expressing the constitutively active CaMKIIγB* mutant in a thymocyte cell line. 166 is a p53-deficient thymoma line expressing CD4, CD8, and a low level of CD3ε (26). We infected 166 cells with a retrovirus expressing Thy1.1 as a reporter gene, with or without CaMKIIγB*. The expression level of CD3ε, CD4, and Thy1.1 was comparable on the infected cell lines (Fig. 5*A*).

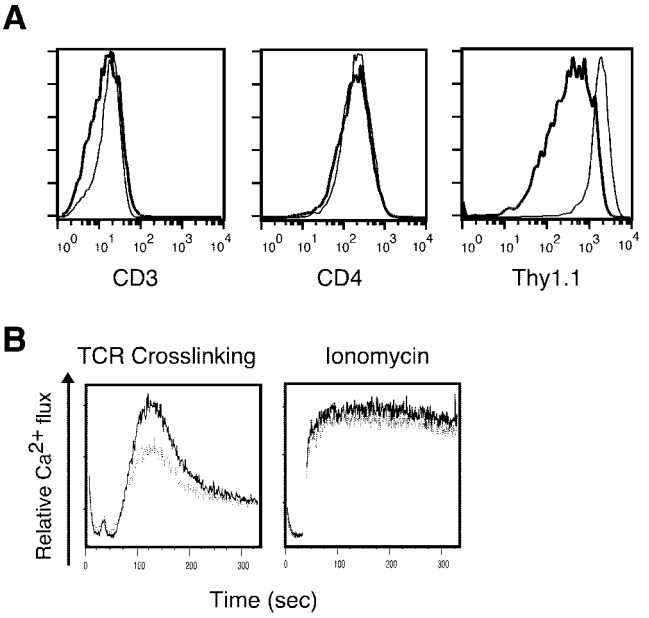

FIGURE 5. Effect of CaMKIIγB*in Ca^{2+} mobilization in a cell line. *A*, The expression of CD3ε, CD4, and Thy1.1 is shown for 166 thymoma cells that were infected with a retrovirus containing an empty vector (thin line) or CaMKIIγB* (thick line). *B*, The retrovirally infected cells were stimulated with anti-CD3 and anti-CD4 (*left*) or ionomycin (*right*) and analyzed for the amount of Ca^{2+} fluxed. The bold line represents cells infected with an empty vector, and the dotted line depicts cells infected with CaMKIIγB*.

Based on expression of Thy1.1, 98% of the cells were infected. However, the expression level of Thy1.1 was slightly lower in the cells cotransduced with CaMKIIγB*, which suggests a toxic effect of high CaMKIIγB* expression, as previously observed for transduced fibroblasts (data not shown). In response to CD3/CD4 cross-linking, the cell line expressing CaMKIIγB* triggered less Ca^{2+} release than the cell line with the empty vector, whereas the response to ionomycin was similar for both lines (Fig. 5*B*). This is consistent with the result observed in the CaMKIIγB* transgenic thymocytes. Therefore, inhibition of TCR signaling by CaMKIIγB* is not due to a developmental defect, but rather it is due to an inhibitory loop involving CaMKII, which can reduce the strength of TCR signaling.

CaMKIIγB inhibitory effect is dominant to the LCKF505 mutant*

CD3ζ-ITAMs are phosphorylated mainly by LCK and, to a lesser extent, by FYN (27). The decrease in phosphorylated CD3ζ and ZAP70 association after TCR engagement in CaMKIIγB* mice suggests an inhibition of LCK activity. Thus, we tested whether a constitutively active form of LCK would rescue the defect in positive selection caused by the CaMKIIγB* transgene. LCK activation occurs by dephosphorylation of the inhibitory Tyr^{505} by the transmembrane PTPase, CD45 (28); hence substituting tyrosine for a phenylalanine at this position (Y505F mutation) results in a constitutively active form of LCK (29). Transgenic mice expressing the LCKF505 mutant (PLGF mice) have a block in T cell development at the DP stage because the cells progress from the DN3 stage to the DP stage without TCR gene rearrangement (30). This developmental problem can be partially rescued by expressing a TCR transgene (30). To determine whether a constitutively active LCK could rescue the signaling defect caused by active CaMKIIγB*, we bred CaMKIIγB* mice with AND;LCKF505 mice. In the latter, expression of CaMKIIγB* still blocked positive selection of $CD4^+$ cells (Fig. 6, *A* and *B*). Therefore, these results suggest that CaMKIIγB* impairs positive selection, even when LCK is constitutively activated.

LCK mutations do not restore Ca^{2+} mobilization

Although a constitutively active LCK did not rescue the defect in selection caused by active CaMKII, it was still possible that CaMKII was altering TCR signaling through LCK. In fact, analysis of the LCK amino acid sequence revealed two motifs containing the CaMKII consensus phosphorylation site: R-X-X-S/T (31). These sites are located at residues 39–42 and 207–210 in LCK. We tested whether CAMKII could interfere with LCK activity through these two sites by mutating the serine and threonine residues of the consensus sites to produce: S42A-LCK, T210A-LCK, and the double mutant, dbl-LCK. These mutants and WT LCK were transduced by retrovirus infection into an LCK-deficient cell line, JCaM. This particular JCaM cell line stably expressed mouse CD4 to provide costimulation to CD3. The LCK constructs also contained Thy1.1 to serve as a reporter for transduction. Stable clones were obtained after Thy1.1 positive selection and tested for the ability to flux Ca^{2+} after CD3 and CD4 stimulation. There was no Ca^{2+} mobilization after TCR activation in JCaM cells in the absence of LCK (data not shown); however, it was restored following expression of WT LCK, or either mutant form of LCK (Fig. 6*C*). This suggests that LCK activity is not affected by mutations at Ser^{42}, Thr^{210}, or both positions. To determine whether CaMKIIγB* would differentially affect signaling in these mutants, CaMKIIγB* was also transduced into each of the mutants using coexpressed Tac (huCD25) as a reporter gene, and the transduced cells were again tested for the release of Ca^{2+}. The

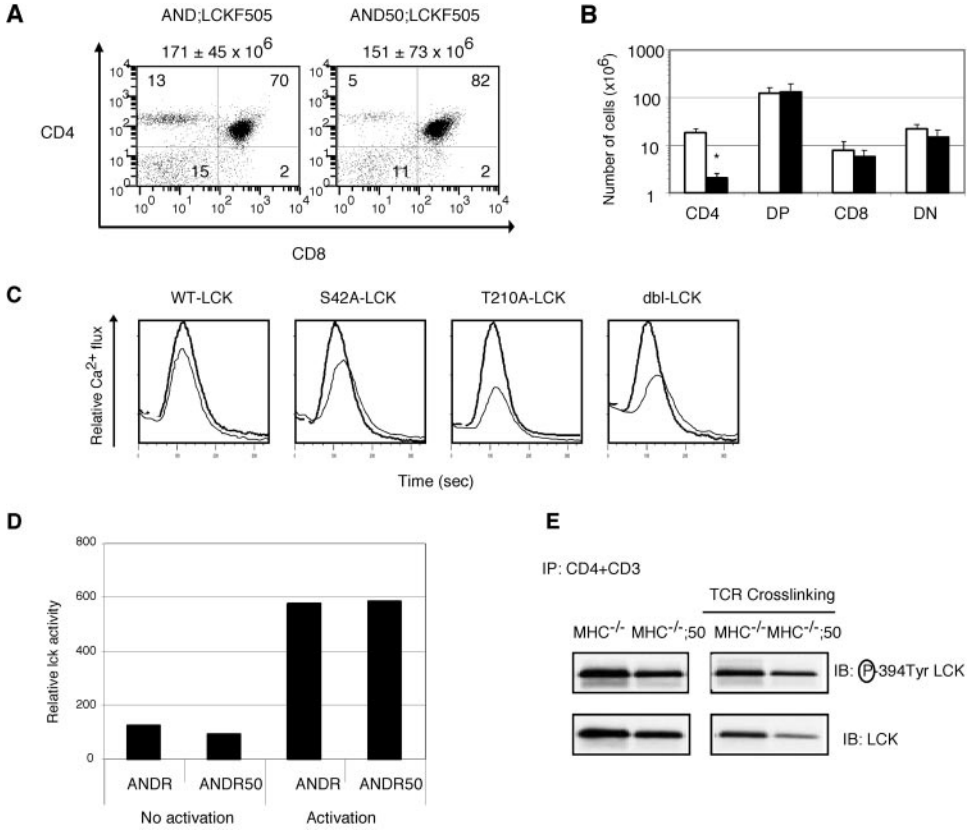

FIGURE 6. CaMKIIγB* impaired positive selection in the presence of a constitutively active form of LCK. *A*, Thymocytes were enumerated and analyzed by flow cytometry for CD4 and CD8 expression. The percentage of each thymocyte subset is indicated. *B*, The absolute numbers of CD4 SP, DP, CD8 SP, and DN cells in AND;LCKF505 white and AND;LCKF505;F50 black mice at 4–6 wk of age are shown ($n = 5$ for AND;LCKF505; $n = 6$ for AND;LCKF505;F50). *, $p < 0.001$, using the two-tailed Student test. *C*, JCaM cells were transfected with WT LCK or a mutant form of LCK (S42A, T210A, or double mutant S24A T210A; bold line). In addition, stable clones were transfected with CaMKIIγB* (thin line). The cells were stimulated with biotin-conjugated anti-CD3 and anti-CD4, cross-linked with streptavidin, and analyzed for Ca^{2+} flux. *D*, LCK was immunoprecipitated from ANDR or ANDR50 thymocytes, before or after 2 min of stimulation, and an in vitro kinase assay was performed with enolase as an exogenous substrate. The graph shows the relative LCK activity determined by densitometry. Data are representative of four separate experiments. *E*, CD4 and CD3ϵ were immunoprecipitated from $MHC^{-/-}$ or $MHC^{-/-}$;F50 thymocytes, before and after stimulation, and analyzed by Western blot with an anti-Tyr^{394} LCK Ab. The blot was reprobed with anti-LCK as a loading control.

presence of CaMKIIγB reduced the amount of Ca^{2+} fluxed in response to anti-CD3 stimulation (Fig. 6*C*). Interestingly, constitutively active CaMKIIγB also reduced the amount of Ca^{2+} flux regardless of whether LCK was mutated at one or both putative phosphorylation sites (Fig. 6*C*). Therefore, CaMKII is not reducing Ca^{2+} flux by directly phosphorylating LCK on either Ser^{42} or Thr^{210}.

LCK activity is not reduced in CaMKIIγB thymocytes*

Finally, to confirm that CaMKII is not inhibiting TCR signaling through LCK activity, we performed an in vitro LCK kinase assay. LCK was immunoprecipitated from AND and ANDR50 thymocytes before and after activation and incubated with the substrate enolase. After activation of thymocytes, LCK activity was not reduced in ANDR50 vs ANDR thymocytes (Fig. 6*D*). We also did not observe a difference in FYN kinase activity (data not shown). LCK is regulated by phosphorylation on multiple residues including Tyr^{505} and Tyr^{394}. Phosphorylation at Tyr^{505} creates an intramolecular association with the SH2 domain, rendering LCK biologically inactive. Phosphorylation of Tyr^{394} overrides inhibition by phosphorylated Tyr^{505}, resulting in kinase activation (32). To confirm that LCK activity was not modified in CaMKIIγB* mice, we looked specifically at LCK Tyr^{394} phosphorylation and found no difference in the amount of active LCK before and after activation in CaMKIIγB* and WT mice (Fig. 6*E*). Therefore, CaMKIIγB* does not affect TCR signaling by inhibiting LCK activity, which suggests that CaMKII targets a different molecule, which is responsible for reduced CD3ζ phosphorylation.

Inhibition of PTPase restored CD3ζ Tyr phosphorylation in CaMKIIγB mice*

Little is known about the mechanism of TCR inactivation, but several PTPases have been implicated in this process (33). It is possible that CaMKII inhibits TCR signaling by activating a PTPase. If this is true, then inhibition of PTPases should restore a normal CD3ζ phosphorylation profile and ZAP70 binding in the presence of CaMKIIγB*. To test this, we stimulated $MHC^{-/-}$ and $MHC^{-/-}$;F50 thymocytes in the presence of pervanadate, a general PTPase inhibitor, and then immunoprecipitated the CD3/CD4 complex to analyze CD3ζ phosphorylation and ZAP70 association. To avoid activation of the T cells before TCR stimulation, we did not pretreat the cells with pervanadate; it was only added in with the streptavidin. As described previously, after TCR stimulation, CD3ζ phosphorylation and ZAP70 association was decreased in $MHC^{-/-}$;F50 thymocytes compared with $MHC^{-/-}$ thymocytes. However, in the presence of pervanadate, CD3ζ phosphorylation, ZAP70 association, and PLCγ phosphorylation were restored in $MHC^{-/-}$;F50 thymocytes (Fig. 7*A*). These data are consistent with a CaMKII-mediated activation of a PTPase, which, in turn, alters

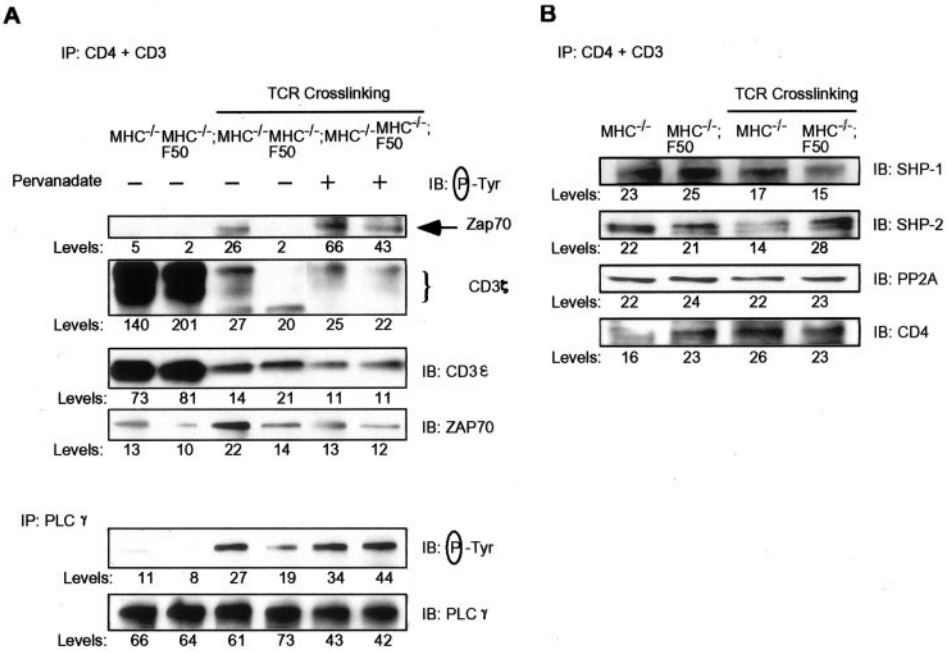

FIGURE 7. CaMKIIγB* recruits a PTPase to the CD3/CD4 complex. *A*, MHC$^{-/-}$ and MHC$^{-/-}$;F50 thymocytes were stimulated with biotin-conjugated anti-CD3ε, anti-CD4, and streptavidin with and without pervanadate. CD3 and CD4 were immunoprecipitated and analyzed by Western blot for anti-phosphotyrosine, ZAP70, and CD3ε, the latter as a loading control. PLCγ was immunoprecipitated and analyzed by Western blot for anti-phosphotyrosine. The membrane was stripped and reprobed with anti-PLCγ as a loading control. *B*, MHC$^{-/-}$ and MHC$^{-/-}$;F50 thymocytes were stimulated as described above. CD3 and CD4 were immunoprecipitated and analyzed by Western blot for SHP-1, SHP-2, PP2A, and CD4, the latter as a loading control.

the proximal TCR signaling events. Furthermore, this PTPase activity is dominant to LCK activity.

To identify this PTPase, we used a program that predicts substrates for kinases based on the primary sequence of a protein kinase catalytic domain (34). This search predicted a consensus phosphorylation site of RQXSFE, and a search of protein databases revealed four PTPases that contained this site: SHP-1; SHP-2; protein phosphatase 2A (PP2A); and protein tyrosine phosphatase, nonreceptor type 2 (PTPN2). Therefore, we investigated the association of these PTPases with the TCR complex following activation in MHC$^{-/-}$;F50 thymocytes compared with MHC$^{-/-}$ thymocytes. After stimulation, the CD3/CD4 complex was immunoprecipitated and analyzed for SHP-1, SHP-2, and PP2A association by Western blot (we could not obtain an Ab that recognized PTPN-2, in mouse thymocytes). There was no difference in SHP-1 or PP2A association with the TCR in MHC$^{-/-}$;F50 and MHC$^{-/-}$ thymocytes, before or after activation (Fig. 7*B*). In WT thymocytes, SHP-2 was associated with the TCR before activation to maintain TCRζ in an unphosphorylated state. Upon stimulation, SHP-2 disassociated from the complex and TCRζ became phosphorylated. However, in MHC$^{-/-}$;F50 thymocytes, SHP-2 remained associated with the TCR complex even after stimulation (Fig. 7*B*). This suggests that CaMKII may be phosphorylating and activating SHP-2, maintaining SHP-2 association with the TCR complex and, in turn, halting TCR signaling. This is an additional, novel mechanism in which T cell signaling is regulated.

Discussion

The elevation of intracellular free Ca^{2+} following TCR engagement is essential for T cell activation. The nature of intracellular Ca^{2+} that is released, whether it is a transient elevation, a repetitive oscillation, or a sustained elevation, depends on the avidity of the TCR for the ligand. Ca^{2+} signals are not a binary switch but contain waves of information that need to be decoded (8, 35). CaMKII, a serine/threonine kinase, is an important biochemical decoder of intracellular Ca^{2+} oscillations (13). In this study, we examined the role of CaMKII in T cell development and found that a constitutively active, Ca^{2+}-independent form of CaMKIIγB reduced positive selection of T cells by maintaining association of SHP-2 with the TCR complex. The persistence of SHP-2 with the TCR complex after activation correlated with a reduction in CD3ζ phosphorylation and ZAP70 association. In turn, PLCγ activation, Ca^{2+} flux, and Erk activation were also reduced following TCR stimulation in cells that express active CaMKII. The decrease in Ca^{2+} mobilization was not due to negative regulation of CRAC channels by CaMKII as it has been reported in a neuron cell line (36), because thymocytes from CaMKIIγB* mice responded to ionomycin with the same intensity as WT thymocytes, whether or not the medium contained extracellular free Ca^{2+}. This work describes a previously unrecognized role of CaMKII in proximal TCR signaling.

CaMKII could be affecting TCR signaling in a number of ways. One possibility is that CaMKII directly inhibits LCK. LCK is responsible for the majority of CD3ζ phosphorylation and contains two CaMKII consensus phosphorylation sites (31). Furthermore, LCK was previously reported to be a substrate of CaMKII (37). Therefore, we were surprised to find that CaMKII did not inhibit TCR signaling by acting directly upon LCK. This was demonstrated by the fact that the presence of a constitutively active LCK did not rescue the defect in positive selection observed in the presence of CaMKIIγB* (Fig. 6, *A* and *B*). In addition, mutation of the two potential CaMKII phosphorylation sites on LCK did not abrogate the defect in Ca^{2+} flux that occurs in the presence of CaMKIIγB* (Fig. 6*C*). Finally, there was no difference in LCK phosphorylation or kinase activity in CaMKIIγB* and WT thymocytes (Fig. 6, *D* and *E*). Together, these data demonstrate that CaMKII does not alter TCR signaling by phosphorylating LCK.

Another possibility is that CaMKII could antagonize TCR signaling by activating and/or recruiting a PTPase to CD3ζ. The fact that the presence of a PTPase inhibitor rescues the signaling defect caused by CaMKIIγB* (Fig. 7*A*) suggests that CaMKII functions upstream of a PTPase. Furthermore, a search of proteins containing the CaMKIIγB* phosphorylation site revealed that four PTPases contained this site. We demonstrated that in the presence of CaMKIIγB*, more SHP-2 was associated with the TCR complex after activation compared with WT thymocytes (Fig. 7*B*). We did not find a difference in association of the other PTPases, SHP-1 or PP2A. SHP-2 is a PTPase containing a tandem SH2 domain that

is expressed ubiquitously. Compared with SHP-1, much less is known about its physiological function in lymphocyte signaling (38). Previous biochemical data suggest that SHP-2 participates in signaling events downstream of Ag receptors and IL-2, physically interacting with a number of cell surface and cytoplasmic signaling proteins in lymphocytes (39). Notably, the interaction of TCR and CTLA-4 results in SHP-2 recruitment and dephosphorylation of CD3ζ (40). Together, these data suggest that CaMKII alters TCR signaling through SHP-2, which dephosphorylates CD3ζ and prevents further signaling through the TCR. Because CaMKII activation depends on the release of Ca^{2+}, this suggests that CaMKII belongs to a regulatory loop where initial signaling events will activate CaMKII, leading to thymocyte activation; however, persistent activation of CaMKII will recruit SHP-2 to the TCR complex and, as a result, modulate TCR signaling. We speculate that the level of SHP-2 recruitment to the TCR will depend on the level of CaMKII-autonomous activity, thus providing a mechanism by which TCR signaling can be fine-tuned by Ca^{2+} oscillations. If there are frequent Ca^{2+} oscillations, more CaMKII subunits will be phosphorylated, as in F50 thymocytes, and more SHP-2 will be recruited, blocking positive selection. In contrast, if a low-affinity ligand induces low or infrequent oscillations, then all of the CaMKII subunits will not be phosphorylated, and SHP-2 will not be maintained at the TCR, thereby allowing positive selection to occur.

To our surprise, we were unable to demonstrate phosphorylation of SHP-2 by CAMKII in an in vitro kinase assay, with immunoprecipitated SHP-2 or rSHP-2 as a substrate (data not shown). However, this may be due to the fact that SHP-2 needs to bind to CD3ζ, or another unidentified protein to be phosphorylated by CaMKII. Moreover, SHP-2 may need to be altered by activation to be phosphorylated by CaMKII. It is also possible that the immunoprecipitated SHP-2 was not phosphorylated, because both the kinase and the substrate were immobilized on beads and they could not interact freely. Alternatively, SHP-2 may not be a direct substrate of CAMKII, and constitutively active CAMKII may increase the recruitment of SHP-2 to the TCR via phosphorylation of another protein. Additional experiments need to be done to address this question. Regardless, the presence of constitutively active CAMKII increases SHP-2 association with the TCR and inhibits positive selection.

It is interesting to note that altered CD3ζ and the lack of ZAP70 recruitment, as well as hyporesponsiveness in IL-2 secretion (41), were also observed in anergic T cells (42). Moreover, a block in inducible IL-2 reporter gene activity was initiated by transfection of CaMKII* in Jurkat T cells (43). Our data suggest the possibility that CaMKII could control IL-2 secretion upstream and downstream of Ca^{2+} signaling by acting on TCR signaling.

We previously reported that, in the presence of the CaMKIIγB* transgene, DP thymocytes have a longer life span in the thymus. From the data presented here, we conclude that this is due to the defect in positive selection. Thymocytes remain at the DP stage until they receive a positive selection signal; thus, in a nonselecting background, the DP thymocytes accumulate until they die by neglect (44, 45). Likewise, the CaMKIIγB* transgene inhibits positive selection and results in an accumulation of DP thymocytes.

It seems unlikely that the previously unsuspected role of CaMKII in a negative-feedback loop, as described here, would be unique to the TCR, given that CaMKII is ubiquitously expressed. CaMKII is highly concentrated at the CNS synapse (46). Moreover, it associates with the *N*-methyl-D-asparate (NMDA) receptor and is necessary for normal synaptic plasticity in pyramidal neurons (47). Binding of CaMKII to the NMDA receptor is potentiated by autophosphorylation at Thr^{286} (48) and may be part of a mechanism modulating the signal strength. Our data suggest that CaMKII modulates the strength of TCR signaling, while regulating a negative feedback loop that could be a more common phenomenon than is currently recognized.

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Disclosures

The authors have no financial conflict of interest.

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