A Pivotal Role for the Multifunctional Calcium/Calmodulin-Dependent Protein Kinase II in T Cells: From Activation to Unresponsiveness[1]

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Stimulation of the TCR leads to an oscillatory release of free calcium that activates members of the calcium/calmodulin-dependent protein kinase II (CaMKII) family. The CaMKII molecules have profound and lasting effects on cellular signaling in several cell types, yet the role of CaMKII in T cells is still poorly characterized. In this report we describe a splice variant of CaMKIIβ, CaMKIIβ'e, in mouse T cells. We have determined its function, along with that of CaMKIIγ, by introducing the active and kinase-dead mutants into activated P14 TCR transgenic T cells using retroviral transduction. Active CaMKII enhanced the proliferation and cytotoxic activity of T cells while reducing their IL-2 production. Furthermore, it induced a profound state of unresponsiveness that could be overcome only by prolonged culture in IL-2. These results indicate that members of the CaMKII family play an important role in regulation of CD8 T cell proliferation, cytotoxic effector function, and the response to restimulation. *The Journal of Immunology,* 2005, 174: 5583–5592.

T he T cell population responds to the introduction of pathogenic agents, while avoiding a productive response to self-Ags (1, 2). The T cell response to foreign pathogens consists of proliferation and differentiation to effector T cells followed by death, memory cell formation, or perhaps terminal differentiation (3–5). Alternatively, recognition of self-Ags may lead to proliferation followed by a state of anergy, and this appears to result from Ag-mediated activation in the absence of the amplifying effects of costimulation (6, 7). Anergy can also result from altered peptide ligands that deliver a suboptimal signal (8). Previous work has shown that the engagement of the TCR results in calcium oscillations (9), and that the frequency or amplitude of calcium oscillations can alter the program of transcriptional activation (10, 11). Yet, at least for lymphocytes, there is little understanding of the mechanisms by which calcium oscillations are interpreted. Calcineurin clearly plays an essential role in T cell activation as well as unresponsiveness in the presence of a calcium ionophore (12–15), but it may not be the only important calcium-calmodulin-activated pathway of signal transduction operative during T cell activation.

Calcium/calmodulin-dependent protein kinase II (CaMKII),[4] a multifunctional serine/threonine kinase, consists of four distinct isoforms (α, β, γ, and δ), and each comprises a family of alternatively spliced variants. A general feature of all CaMKII isoforms is their ability to decode the frequency of calcium oscillations by retaining calcium-independent kinase activity subsequent to the initial stimulation. The α and β isoforms are expressed abundantly in neurons, while the γ and δ isoforms are more widely expressed (16). CaMKII exists as a holoenzyme consisting of 6–12 subunits with each subunit composed of an N-terminal catalytic domain, a regulatory domain, a variable domain, and a C-terminal association domain. The inhibitory region and calmodulin-binding site are within the regulatory domain. Upon a rise in calcium, four molecules of calcium interact with one molecule of calmodulin to form a calcium/calmodulin complex that binds to targets of calcium signaling pathways. Once calcium/calmodulin binds to the calmodulin-binding site of CaMKII, the inhibitory region is disrupted, activating kinase activity and promoting the phosphorylation of a key threonine residue (Thr[287] of CaMKIIβ, γ, δ) by an adjacent subunit. Phosphorylation of Thr[287] is sufficient to disrupt the inhibitory region even after calmodulin dissociates and results in autonomous CaMKII activity (16). A previous report showed that alternatively spliced forms of CaMKIIβ exhibited differential responses to the frequency of calcium oscillations (17), suggesting a mechanism for cells to modulate their calcium sensitivity.

CaMKIIα has been shown to play an important role in long-term potentiation and permanent memory in the brain, as ablation or a Thr[286]→Ala mutation of CaMKIIα caused defects in long-term potentiation and spatial learning (18, 19). However, little information is available regarding the expression and function of CaMKII in T cells. Previous studies showed that constitutively active CaMKIIγB, CaMKIIγB T287D, blocked IL-2 and IL-4 promoter activities in Jurkat T cells, suggesting a role of CaMKIIγB in regulation of cytokine production (20, 21). We have found that

transgenic mice expressing CaMKIIγB T287D showed enhanced T cell activation and an increase in the proportion of memory T cells (22). These studies suggested that CaMKIIγB could be involved in differentiation as well as memory formation in T cells.

To characterize the role for CaMKII in T cell activation, we first set out to establish the identity of CaMKII isoforms present in T cells. In addition to CaMKIIγ and CaMKIIδ, we found that the CaMKIIβ′e splice variant, previously only found as a fetal isoform, was expressed in both CD4 and CD8 T cells. We then studied the effects of CaMKII activation in T cells by introducing active or kinase-dead mutants into Ag-activated, CD4 or CD8 T cells by retroviral transduction. We showed that expression of two isoforms of active CaMKII resulted in very similar changes to activated CD8 T cells, including enhanced proliferation and cytotoxic activity, diminished IL-2 production, and the induction of unresponsiveness.

Materials and Methods

Mice

The mice were bred and maintained at the University of California at San Diego animal facility. TCR transgenic CD8 T cells were harvested from P14 mice (23) and OT-I mice (24).

Media and Ab

Unless otherwise stated, all cell culture was performed in RPMI 1640 (Invitrogen Life Technologies) supplemented as described previously (25). EHAA (Invitrogen Life Technologies) was supplemented as RPMI 1640 but without nonessential amino acids. 293 T cells were cultured in DMEM high glucose containing 10% FBS. Anti-β-tubulin Cy3 was purchased from Sigma-Aldrich, microbead-conjugated Abs from Miltenyi Biotec, and all other Abs from eBioscience.

RT-PCR and DNA sequencing

To determine the expression of CaMKIIβ in T cells, total RNA from purified T cells was subjected to cDNA synthesis by using a ThermoScript RT-PCR kit (Invitrogen Life Technologies) per the manufacturer's instructions and cDNA synthesized from 200 ng of RNA was amplified with CaMKIIβf1 (ggatttgcgggaacgccagg) and CaMKIIβr1 (gctggtgatggc cgagctg). The PCR mix contained 40 μl of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl$_2$, 188 μM dNTPs, and 1 U of Taq DNA polymerase. The amplifications consisted of 45 cycles of 30 s at 94°C, 55 s at 62°C, and 1 min at 72°C followed by 5 min at 72°C. The amplified DNA was sequenced with CaMKIIβr1. Because a splice variant(s) of CaMKIIβ without exon IX was detected (see Fig. 1), the cDNA was amplified with CaMKIIβf2 (caaaaa cagctcggccatcaccagc) and CaMKIIβr2 (ctcactgcagcggggccactggag) to identify the variant(s). The RT-PCR condition was the same as above except 2 mM MgCl$_2$ and 64°C annealing temperature were used. The PCR product was sequenced by using T7 (gtaatacgactcactatagggc) and M13(ggaaac agctatgaccatg) primers. DNA sequencing identified expression of CaMKIIβ′e but not CaMKIIβe. To determine the possible presence of CaMIIβe, the cDNA was amplified with CaMKIIβf1 and CaMKIIβe (atggataacggtgg tttgagg). CaMKIIβf1 and CaMKIIβm (actgagttcaggatgtcagag) were used to detect CaMKIIβ$_3$. The amplification conditions of these two primer sets were the same as that of CaMKIIβf1 and CaMKIIβr1. The PCR product was sequenced with CaMKIIβm and CaMKIIβvL (ccagacaaacagcacaaaa). β-actin primers (sense: gtgggccgctctaggcaccaa and antisense: ctctttgatgt cacgcacgat) were used to determine cDNA quality. Ten microliters of the PCR products was resolved on a 1% agarose gel.

Vector construction

To study the localization of CaMKIIβ′e, yellow fluorescent protein (YFP) was tagged at N terminus of CaMKIIβ′e (26). Because mouse and rat CaMKIIβ′e are highly homologous (27, 28), rat CaMKIIβ′e (a gift from Dr. H. Schulman, Stanford University School of Medicine, Stanford, CA) was used. To clone CaMKIIβ′e into pEYFP-C1 vector (BD Clontech), CaMKIIβ′e in pSRα was digested with EcoRI, filled in with DNA polymerase Klenow fragment (New England Biolabs), and ligated with pEYFP-C1 which was digested with XmaI and filled in with the Klenow fragment.

CaMKIIβ′e T287D and CaMKIIβ′e K43M were generated by quick change site-directed mutagenesis and cloned into retrovirus vector MiT (a gift from Dr. P. Marrack, University of Colorado Health Sciences Center,

Denver, CO) (29) as follows: CaMKIIβ′e mutants in pSRα were digested with EcoRI, cloned into pBlue Script II SK⁻ vector to gain restriction sites, and then ligated with a ClaI- and NotI-digested MiT vector. Human CaMKII γB T287D in pSRα (a gift from Dr. H. Schulman) was amplified by PCR with primers containing NotI and ClaI restriction sites (gggcggc-cgcatggccaccaccgccacctgc and aaatcgatggctgagctcactgcagcggtgc) and then cloned into a NotI- and ClaI-digested MiT vector.

Localization of CaMKIIβ isoforms

To examine localization of CaMKIIβ in T cells, D10 T cells were electroporated with GFP-CaMKIIβ (a gift from Dr. K. Shen, University of California, San Diego, CA) or YFP-CaMKIIβ′e, fixed with 2% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and stained with 4′,6′-diamidino-2-phenylindole (DAPI) or anti-β-tubulin Cy3. To determine whether CaMKIIβ isoforms translocate after Ag stimulation, D10 T cells expressing GFP-CaMKIIβ or YFP-CaMKIIβ′e were stimulated with LK cells which had been pulsed with conalbumin or pigeon cytochrome c (PCC; as a negative control) and labeled with Cy5 (Amersham). Live cell imaging was performed as described previously (30).

Retroviral transduction

To introduce genes into T cells by retroviruses, pooled spleen and lymph node cells from TCR transgenic mice were cultured in EHAA, and stimulated with specific peptides 1 day before spin infection. P14 and OT-I T cells were stimulated with 0.2 μM gp33 (KAVYNFATC; Genemed) and 10 nM OVA 257 (SIINFEKL; Calbiochem), respectively. Retroviruses were prepared by cotransfecting 293 T cells with ψ Eco plasmid and retrovirus vector as described previously (31). Viral supernatants were supplemented with 10 mM HEPES, pH 7.2, and 8 μg/ml polybrene. A total of 2.5×10^6 activated cells in 2 ml of viral supernatants were plated per well of a 24-well plate and spun for 3 h at 32°C.

Cell purification and staining

To isolate T cells, pooled spleen and lymph node cells from naive C57BL/6 mice were stained with MACS CD4 or CD8 microbeads and purified by immunomagnetic sorting. For purification of Thy1.1 cells, retrovirus-transduced cells were separated with lympholyte (Cedarlane Laboratories), stained with biotin-conjugated anti-Thy1.1 Ab and streptavidin microbeads, and subjected to immunomagnetic sorting. To isolate CaMKIIγB T287D^{low} and CaMKIIγB T287D^{high} T cells, the transduced T cells were stained with anti-Thy1.1 FITC and anti-CD8 PE and sorted by FACSVantage (BD Biosciences).

For staining with CFSE (Molecular Probes), 1×10^7 cells were incubated with 2 μM CFSE for 10 min in PBS containing 0.1% BSA. To examine retrovirus-transduced cells, the cells were stained with anti-CD8 PerCP, anti-Thy1.1 PE, anti-CD25 FITC, and analyzed by FACS using a FACSCalibur (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star).

To determine IL-2 production, 1×10^6 cells were plated per well of a 24-well plate, stimulated with 50 ng/ml PMA (Calbiochem) and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h. At the last 2 h of stimulation, 1 μl of Golgiplug (BD Pharmingen) was added per well. Intracellular IL-2 staining was performed by using the Cytofix/Cytoperm kit (BD Pharmingen) per the manufacturer's instructions. Alternatively, Thy1.1-purified cells were stimulated with PMA and ionomycin in the absence of Golgiplug. Culture supernatants were collected at 4 h after stimulation and evaluated with a mouse IL-2 ELISA kit (eBioscience).

Cytotoxicity assay

To prepare target cells, the TAP-2-deficient cell line RMA-S (H-2^b) was pulsed with 5 μM gp33. Cell-mediated cytotoxicity was determined as described previously (32).

APC preparation and Ag restimulation

To prepare APC, C57BL/6 spleen cells were incubated with anti-Thy1 Ab (T24) and then treated with rabbit complement (Cedarlane Laboratories) and 50 μg/ml mitomycin C (Sigma-Aldrich). For Ag restimulation, Thy1.1-purified T cells were either untreated in media for 3 days or treated with 50 U/ml rIL-2, a gift from Cetus, for 1–2 wk. A total of 4×10^4 T cells and 1.2×10^5 APCs in 0.2 ml of media were added to microtiter plates in the presence of various doses of gp33. The cells were pulsed with [³H]thymidine (PerkinElmer) for 14–16 h at 2 days after restimulation, harvested with a cell harvester (Tomtec), and counted by a Betaplate liquid scintillation counter (Wallac). Results are expressed as the mean ± SD of triplicate samples.

Western blot

To determine CaMKIIγB expression, purified T cells were lysed in high salt lysis buffer, and subjected to Western blot as described previously (22) except protein was transferred to Immobilon-P membrane (Millipore) and visualization and quantification were performed by using ECL plus and a Typhoon 9400 Phosphoimager (Amersham). For loading control, the membrane was incubated with anti-ERK1,2 Ab (Zymed Laboratories) and goat anti-rabbit IgG HRP (Vector Laboratories).

Results

CaMKIIβ′e identified in CD4 and CD8 T cells

To understand the roles that CaMKII can play in T cell activation, we identified the isoforms and their splice variants that are expressed in T cell subsets. Similar to published reports using cell lines (33, 34), we confirmed that several splice variants of CaMKIIγ and CaMKIIδ are expressed (our unpublished data), but in addition, we found that CaMKIIβ, an isoform previously thought to be restricted to neurons, is also expressed in T cells. Although previous studies have shown that CaMKIIβ plays an important role in neuronal plasticity (35), a function for CaMKIIβ outside the nervous system is unknown.

To analyze the expression of CaMKIIβ, CD4 and CD8 T cells from naive C57BL/6 mice were purified and the isolated RNA subjected to RT-PCR. CaMKIIβf1 and CaMKIIβr1 primers spanning the catalytic and variable regions of CaMKIIβ (Fig. 1A) were used to amplify all of the splice variants of CaMKIIβ (28). As shown in Fig. 1B, full-length CaMKIIβ was not detected in CD4 or CD8 T cells; however, a smaller PCR product was specifically amplified in both, suggesting expression of a splice variant(s) of CaMKIIβ in the T cells. Direct sequencing of the PCR product verified the expression of one or more of the splice variants of CaMKIIβ that lack exon IX in CD4 and CD8 T cells (Fig. 1A). To provide further evidence that the splice variant of CaMKIIβ was from T cells and not from non-T cells in the purified samples, we also examined its expression in D10 and AE7 T cell lines. The same sized band was detected in both T cell lines (data not shown), indicating that it was unlikely to be due to contaminating non-T cells.

Because CaMKIIβf1 and CaMKIIβr1 primers amplified both CaMKIIβe and CaMKIIβ′e, CaMKIIβf2 and CaMKIIβr2 primers spanning the variable and association regions (Fig. 1A) were used for RT-PCR to distinguish these two splice variants. The sequences of 20 clones (CD4 and CD8 T cells, 10 each) from the RT-PCR products indicated that CaMKIIβ′e but not CaMKIIβe was expressed (Fig. 1B and data not shown). In addition, the absence of CaMKIIβe and CaMKIIβ in T cells was further confirmed with CaMKIIβf1 and CaMKIIβe primers (Fig. 1B). To determine whether the CaMKIIβ₃ splice variant is expressed in T cells, RT-PCR was conducted using CaMKIIβf1 and CaMKIIβm primers. The results from the PCR and subsequent sequencing of the purified PCR products indicate that the CaMKIIβ₃ splice form is expressed (Fig. 1B and data not shown). The fact that we did not find this variant when sequencing the products of CaMKIIβf2 and CaMKIIβr2 primers indicates that it is present at very low levels. It was not studied further. Although CaMKIIβ′e has been identified in embryonic brain (28), its expression in T cells has not been previously reported. In this study, we characterized the localization and function of CaMKIIβ′e and CaMKIIγB in T cells.

Localization of CaMKIIβ isoforms in the T cells

CaMKIIβ has been shown to be constitutively associated with F-actin and to slowly dissociate upon glutamate stimulation of the N-methyl-D-asparate-glutamate receptor (36). We thus determined the localization of CaMKIIβ′e in T cells before and after TCR
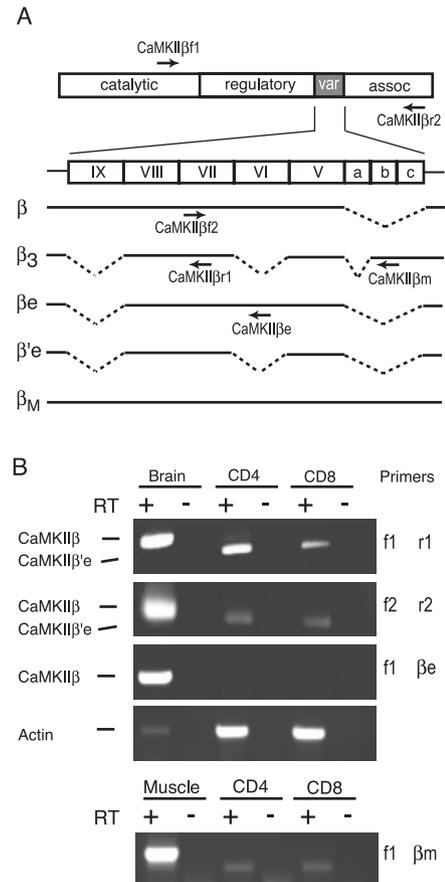

FIGURE 1. Identification of CaMKIIβ′e and CaMKIIβ₃ in CD4 and CD8 T cells. A, Schematic of CaMKIIβ structure is presented. The nomenclature of exons in the variable region is as described previously (62) and the dashed lines represent deleted exons in each splice variant. The arrows show approximate position of the primers used for RT-PCR. B, CaMKIIβ′e and CaMKIIβ₃ were detected in T cells by RT-PCR and DNA sequencing as described in *Materials and Methods*. The primer sets used to detect various splice variants are listed next to each gel. Primer f1 represents CaMKIIβf1; r1, CaMKIIβr1; etc. CD4 and CD8 T cells were isolated from pooled spleen and lymph node cells of naive C57BL/6 mice by immunomagnetic sorting. The purities of the sorted CD4 and CD8 T cells were 93 and 97%, respectively.

stimulation. To examine this, CaMKIIβ′e was tagged with YFP (YFP-CaMKIIβ′e) and expressed in D10 T cells. The expression of YFP-CaMKIIβ′e was verified by Western blot (data not shown). For comparison, we ectopically expressed GFP-CaMKIIβ in D10 T cells. While YFP-CaMKIIβ′e was found in the T cell cytoplasm (Fig. 2A), a substantial amount of GFP-CaMKIIβ formed a bright cytoplasmic granule (Fig. 2B). Anti-β-tubulin staining of GFP-CaMKIIβ-transfected D10 T cells revealed that the bright granule was due to an association of GFP-CaMKIIβ with the microtubule-organizing center (MTOC) (Fig. 2C), consistent with studies showing that CaMKII associates with microtubules in neuronal extracts (37). Upon TCR stimulation, GFP-CaMKIIβ dissipated from the MTOC to the cytoplasm in a manner reminiscent of CaMKIIβ moving from the actin cytoskeleton to the postsynaptic density upon the release of free calcium. The dissipation was Ag-specific, as it only occurred when the transfected D10 T cells were stimulated with conalbumin-pulsed but not PCC-pulsed APC. In contrast, CaMKIIβ′e was not associated with the MTOC and did not translocate to a different subcellular compartment after interaction with conalbumin-pulsed APC. In particular, it was not localized to the point of contact between the T cell and
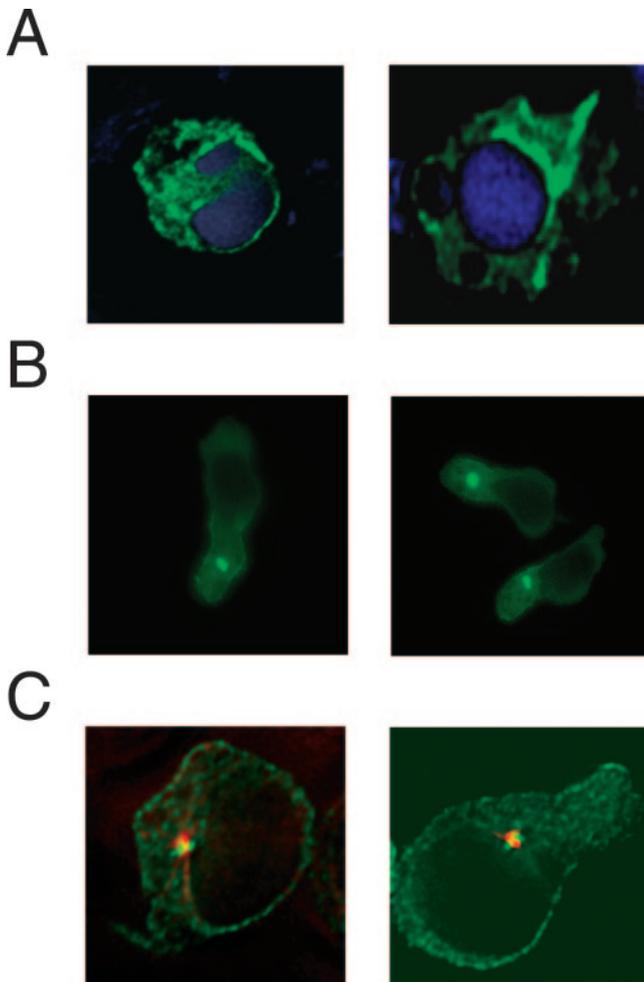
FIGURE 2. Localization of CaMKIIβ isoforms in D10 T cells. D10 T cells were transfected with (*A*) YFP-CaMKIIβ'e and stained with DAPI. D10 T cells were transfected with GFP-CaMKIIβ and examined by (*B*) live cell imaging or stained with (*C*) anti-β-tubulin Cy3. Each experiment was done three times.

the APC (data not shown). We conclude that T cells specifically express a form of CaMKIIβ that does not associate with the cytoskeleton or the MTOC.

Constitutive expression of active CaMKIIβ'e promotes proliferation but not survival of CD8 T cells

To study the function of CaMKIIβ'e in T cells, active (CaMKIIβ'e T287D) and kinase-dead (CaMKIIβ'e K43M) mutants were cloned into the retrovirus vector MiT, and introduced into activated CD8 T cells by retroviral transduction. Because the retrovirus vector MiT contains Thy1.1 as a reporter of the transgene, we were able to trace T cells expressing the transgene by staining with anti-CD8 and anti-Thy1.1. We first examined its effect on CD8 T cell proliferation by CFSE labeling. We note that the retroviral transduction necessarily occurs subsequent to the initial TCR-mediated stimulation, so this protocol examines the effects of CaMKII on the course of proliferation subsequent to the initial activation. As shown in Fig. 3*A*, the distribution of P14 T cells expressing active CaMKIIβ'e was shifted by approximately three additional rounds of cell division. In contrast, the T cells expressing either a wild-type or a kinase-dead mutant showed only a modest change in the distribution of cell divisions. The enhanced T cell division was also observed by enumerating the number of Thy1.1 cells over time (Fig. 3*B*). These results indicated that active CaMKIIβ'e can
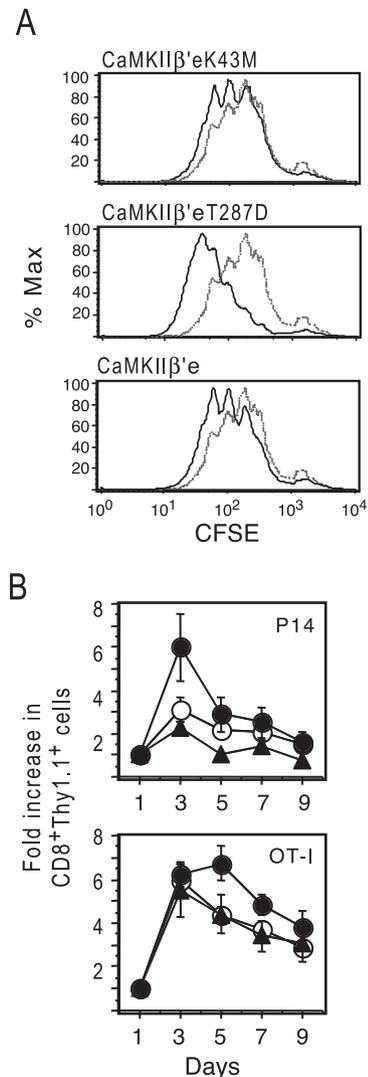

FIGURE 3. Promotion of CD8 T cell proliferation but not survival by active CaMKIIβ'e. *A*, P14 T cells were labeled with CFSE and transduced with retroviruses expressing CaMKIIβ'e K43M, CaMKIIβ'e T287D, or CaMKIIβ'e. CFSE analyses were determined at 3 days after spin infection. In all histograms, gray lines represent P14 T cells expressing MiT vector and dark lines represent the T cells expressing wild-type or CaMKIIβ'e mutants. The relative numbers of (*B*) P14 T cells and OT-I T cells expressing MiT (○), CaMKIIβ'e K43M (▲), and CaMKIIβ'e T287D (●) were determined by trypan blue exclusion and FACS analyses at various days after spin infection. The number of Thy1.1[+] CD8 T cells at each time point was divided by that of day 1 to normalize the transduction efficiency.

play a role in T cell proliferation and initial accumulation. Interestingly, while active CaMKIIβ'e enhanced CD8 T cell proliferation, it did not promote cell survival at later time points (Fig. 3*B*), as the T cell number dropped dramatically at 5 days after spin infection and remained similar to those expressing the vector control and the kinase-dead mutant. To ensure that the effect was not restricted to P14 T cells, we also introduced the active and kinase-dead mutants into OT-I T cells by retrovirus. Likewise, active CaMKIIβ'e enhanced proliferation but not survival of OT-I T cells. However, the enhanced T cell proliferation was detected at 5 days instead of 3 days after spin infection. The proliferative effect of CaMKIIβ'e on CD4 T cells was determined by introducing the active mutant into PCC-stimulated AND transgenic T cells; however, we found no consistent stimulation of proliferation (data not shown).

The enhanced CD8 T cell proliferation is not due to cytokine production

Previous studies have shown that active CaMKIIγB blocked IL-2 and IL-4 production (20, 21), and this would seem to counter the observed increase in proliferation. To examine production of cytokines in retrovirus-transduced T cells, we subjected the cells to intracellular IL-2 staining and IL-2 ELISA as depicted in Fig. 4*A*. P14 T cells expressing active CaMKIIβ′e produced less IL-2 than those expressing the vector control such that for a given threshold, fewer cells were scored as positive (39 vs 52%). A decrease in IL-2 production in T cells expressing active CaMKIIβ′e was also detected by IL-2 ELISA (Fig. 4*A*, *lower panel*). In contrast, P14 T cells expressing the kinase-dead mutant and the vector control produced comparable amounts of IL-2. Furthermore, we examined CD25 expression on P14 T cells expressing CaMKIIβ′e mutants to see whether the enhanced proliferation was due to an increase in IL-2 consumption. Similar levels of CD25 were expressed on T cells transduced with active CaMKIIβ′e, kinase-dead mutant, and vector control (data not shown), indicating that the enhanced proliferation was not due to an increase in IL-2 consumption.

Although active CaMKIIβ′e resulted in an inhibition of IL-2 production, it could have up-regulated other cytokines to stimulate CD8 T cell proliferation. Because receptor occupancy, and thus proliferation, is proportional to the concentration of free cytokines, we would expect to detect enhanced T cell proliferation upon addition of the culture supernatants from CD8 T cells expressing active CaMKIIβ′e. To test this, culture supernatants of P14 T cells expressing various CaMKIIβ′e mutants were collected at 2 and 3 days after spin infection and then added to activated P14 T cells to determine whether they could enhance T cell proliferation. The experiment presented in Fig. 4*B* reveals that the addition of the culture supernatants had no effect on T cell proliferation, whereas the addition of IL-2 enhanced DNA synthesis 36-fold. Thus, the enhanced proliferation is not due to excess growth factors, although we cannot rule out the possibility of a small amount of growth factor that is biologically undetectable in this assay.

Constitutive expression of active CaMKIIβ′e induces unresponsiveness to Ag restimulation

The formation of effector T cells capable of cytolytic activity is preceded by proliferation, but in many cases, the establishment of anergy as a mechanism of peripheral tolerance is also preceded by proliferation. Such tolerance can occur under conditions of suboptimal stimulation, and it is characterized by partial deletion of the responding population with the remaining cells exhibiting altered effector functions and a diminished response (38–41). The requirements for productive CD8 T cell activation include an Ag stimulus, a second costimulatory signal, and a third signal that may take the form of IL-12 (42).

To examine the responsive state of T cells subsequent to CaMKIIβ′e expression, P14 T cells expressing the retroviral constructs were sorted for Thy1.1 expression and subjected to Ag restimulation using a protocol depicted in Fig. 5*A*. The consistent result was that CD8 T cells expressing active CaMKIIβ′e failed to respond to Ag restimulation, while the T cells expressing the vector control or the kinase-dead mutant showed an Ag dose-dependent proliferation (Fig. 5*A*). This result suggests that CD8 T cells transduced with active CaMKIIβ′e were refractory to Ag restimulation, and we propose that this lack of response to restimulation may simulate one of the physiological states of T cell unresponsiveness. For instance, it may reflect either the anergy associated with the absence of costimulation, or a state of terminal differen-
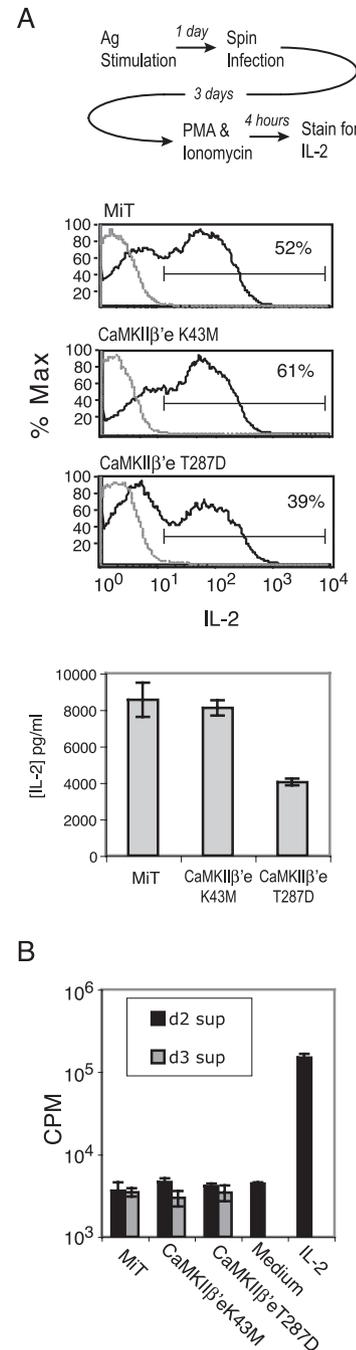

FIGURE 4. The enhanced proliferation of CD8 T cells expressing active CaMKIIβ′e was not due to an increase in cytokine production. *A*, IL-2 production of P14 T cells expressing various CaMKIIβ′e mutants was determined by intracellular staining (*upper panel*) and IL-2 ELISA (*lower panel*) as described in *Materials and Methods*. In all histograms, gray lines represent control IgG2b staining and dark lines represent IL-2 staining. *B*, Proliferation of P14 T cells was determined in the presence of culture supernatants collected from P14 T cells expressing CaMKIIβ′e mutants. The culture supernatants were collected at 2 and 3 days after spin infection and added to the P14 T cells which had been activated with 0.2 μM gp33 for 1 day. For a positive control, 20 U/ml IL-2 was added to test the responsiveness of the cells.

tiation exhibited by productively activated T cells that nonetheless are not promoted to memory cells.

A previous report showed that CD4 T cell anergy was reversed by prolonged culture in IL-2 (43), and this prompted us to determine whether unresponsiveness in CD8 T cells can be likewise
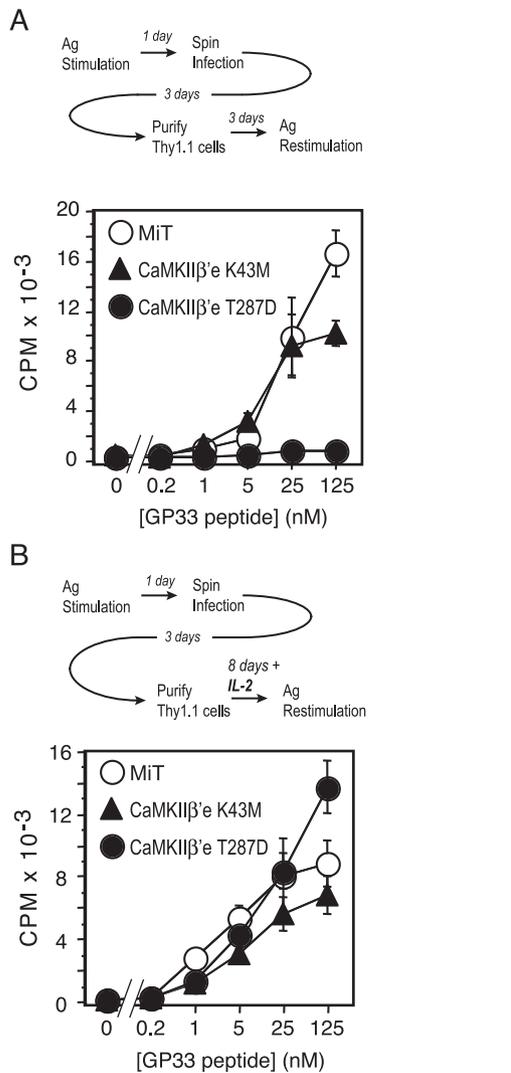
FIGURE 5. Expression of CaMKIIβ'e by retroviral transduction of activated T cells caused profound unresponsiveness which was reversed by prolonged culture in IL-2. P14 T cells expressing various CaMKIIβ'e mutants were treated (*A*) without or (*B*) with 50 U/ml IL-2 after purification of the Thy1.1 population. The stimulation protocol is delineated as a flow chart at the top of each figure. The cells were pulsed with [^{3}H]thymidine at 2 days after the restimulation as described in *Materials and Methods*.

FIGURE 6. T cell unresponsiveness was not reversed by PMA and ionomycin or by the addition of IL-2. P14 T cells expressing various CaMKIIβ'e mutants were treated with 10 ng/ml PMA and 1 μM ionomycin or with 50 U/ml IL-2 during Ag restimulation. The stimulation protocol is depicted as a flow chart. The cells were pulsed with [^{3}H]thymidine at 2 days after the restimulation.

overcome. T cells expressing various CaMKIIβ'e mutants were treated with exogenous IL-2 and then subjected to Ag restimulation as depicted in the flow chart of Fig. 5*B*. As shown, the ability of CD8 T cells expressing active CaMKIIβ'e to proliferate was restored by 8 days of culture in IL-2. Initial experiments showed that 3 days of culture in the presence of IL-2 was not sufficient to overcome unresponsiveness (data not shown). The IL-2 effect was not due to an outgrowth of cells, since there was very little proliferation after 3 days in culture, and it was not due to selective loss of cells expressing the retroviral construct, since the T cells expressing Thy1.1 reporter gene increased in percentage after Ag stimulation (data not shown).

We next asked whether the unresponsive T cells could be persuaded to respond by a stimulus that bypasses the proximal TCR-mediated signals. As depicted in Fig. 6, T cells were stimulated with optimal concentrations of PMA and ionomycin, and analyzed for proliferation measured by the incorporation of [^{3}H]thymidine after 2 days of reactivation. As shown, PMA and ionomycin stimulated cells transduced with the control vector or kinase-dead ver-
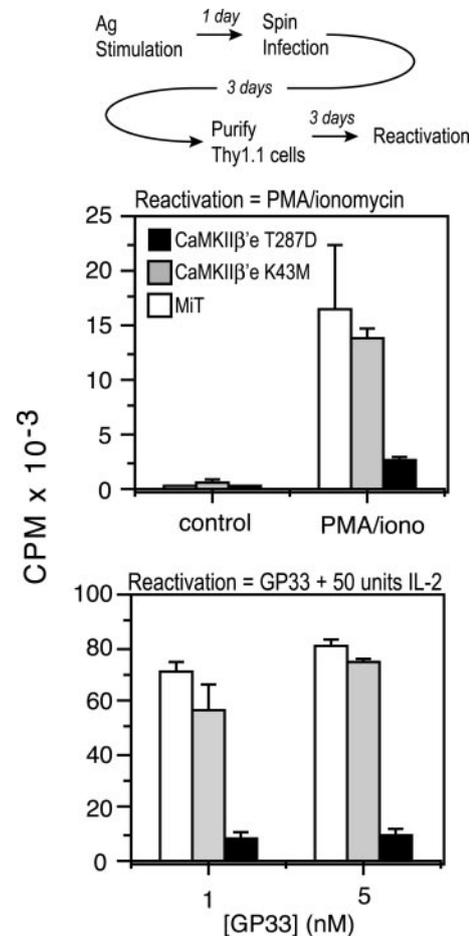
sion of CaMKIIβ'e, while there was little stimulation in the population transduced with active CaMKIIβ'e. Likewise, the addition of IL-2 did not rescue Ag-mediated proliferation. This refractory state is similar to that found for male Ag-specific T cells stimulated under tolerizing conditions (41), but it would appear to differ from activation-induced nonresponsiveness (AINR) in which CD8 T cells are activated in the absence of IL-2 (44, 45).

CaMKIIγB promotes proliferation or unresponsiveness depending on the levels of activation

We previously published that *CaMKIIγB T287D (γB*)*, expressed as a transgene, enhanced T cell proliferation and caused a substantial increase in the number of Ag-reactive memory cells (22). This presented two possibilities. Either CaMKIIβ and CaMKIIγ have different effects on T cell activation, or the effect of these molecules depends on the timing or level of activation. The γB* transgene was present throughout activation, whereas in the experiment presented above, active *CaMKIIβ'e* was present 24 h after the initial activation and expressed from a retroviral promoter. To investigate this further, we compared the proliferation and reactivation of T cells from *P14;γB** double transgenic mice with P14 T cells transduced at 24 h after activation with MiT vector containing CaMKIIγB T287D (Fig. 7). Consistent with data previously shown, the presence of the γB* transgene caused T cells to undergo more rounds of proliferation; however, these T cells showed
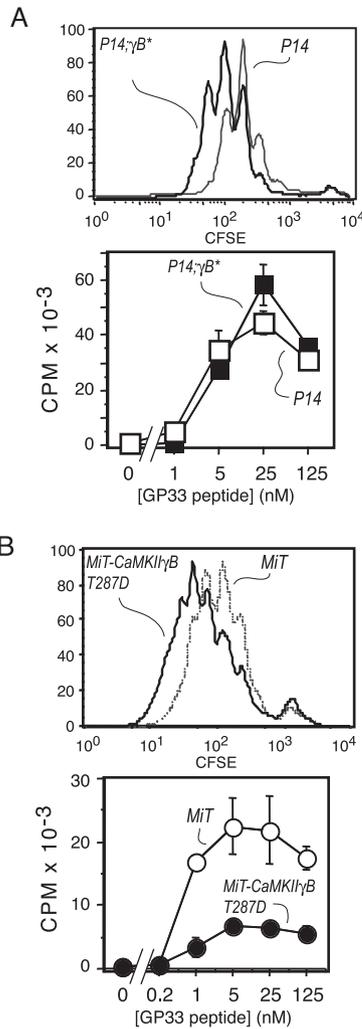
FIGURE 7. P14;γB* T cells but not CaMKIIγB T287D-transduced P14 T cells were responsive to Ag restimulation. *A*, T cells from P14;γB* mice were hyperproliferative after primary Ag stimulation and capable of responding to restimulation. T cells were labeled with CFSE and stimulated with 0.2 μM gp33 for 4 days (*upper panel*). At 7 days after stimulation, T cells were restimulated with gp33 as described in *Materials and Methods* (*lower panel*). *B*, CaMKIIγB T287D-transduced P14 T cells were hyperproliferative but unresponsive to restimulation. P14 T cells were labeled with CFSE and transduced with CaMKIIγB T287D. CFSE analysis was determined at 4 days after spin infection (*upper panel*). Ag restimulation (*lower panel*) was performed as described in the flowchart of Fig. 5*A*. The purities of sorted Thy1.1 cells for MiT- and CaMKIIγB T287D-transduced P14 T cells were 84 and 93%, respectively.

no deficiency in restimulation after 7 days in culture (Fig. 7*A*). In sharp contrast, the transduction of active *CaMKIIβB* after activation induced enhanced proliferation and a strong state of unresponsiveness (Fig. 7*B*). We conclude that activated CaMKIIβ′e and CaMKIIγB have similar effects on T cell physiology, and these effects depend on either the timing or level of activation.

To distinguish between timing and levels of activation, two types of experiments were performed. In the first, T cells from *P14* or *P14;γB** mice were transduced with the MiT vector with or without *CaMKIIγB T287D*. If timing is the most important parameter, then the γB* transgene should overcome the loss of reactivity associated with the transduction of *CaMKIIγB T287D* at 24 h after the initial activation. As shown in Fig. 8*A*, regardless of whether γB* was present, the transduction of *CaMKIIγB T287D* caused a loss of Ag-induced reactivity. Western blot analysis showed that
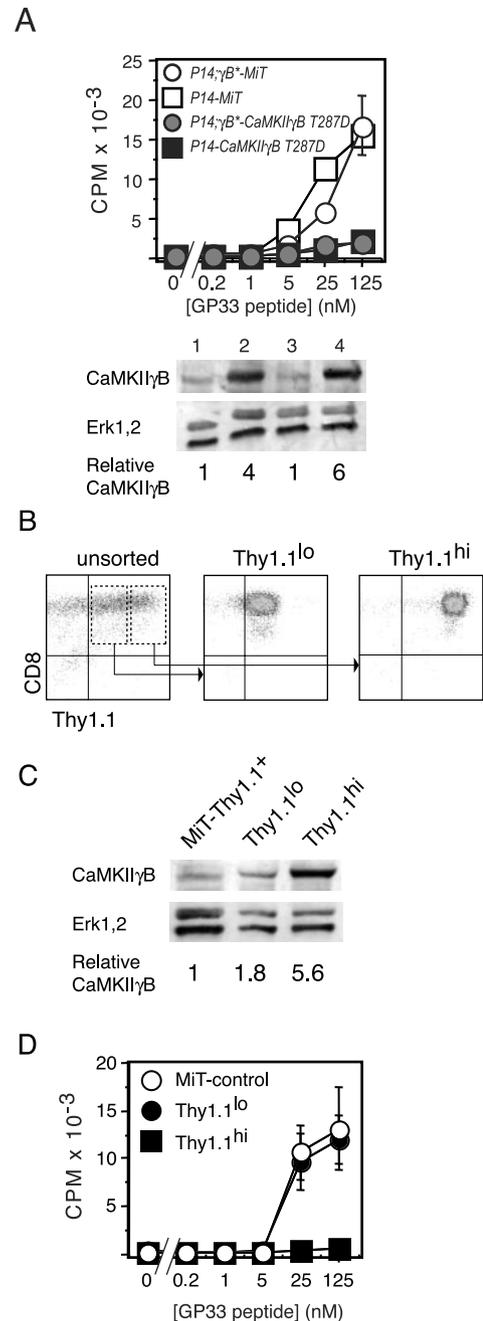

FIGURE 8. T cells expressing a low but not a high level of CaMKIIγB T287D responded to Ag restimulation. *A*, P14;γB* T cells transduced with CaMKIIγB T287D were unresponsive to restimulation. Restimulation was performed as described in Fig. 5*A*. CaMKIIγB expression was determined by Western blot as described in *Materials and Methods*. P14 T cells were transduced with MiT (1) or CaMKIIγB T287D (2); P14;γB* T cells were transduced with MiT (3) or CaMKIIγB T287D (4). To calculate relative CaMKIIγB, the ratio of CaMKIIγB to ERK2 expression (a control for protein loading) in each lane was divided by that of *lane 1*. *B*, CaMKIIγB T287D- transduced P14 T cells were sorted for Thy1.1^low and Thy1.1^high populations by FACS. MiT-transduced P14 T cells were purified by immunomagnetic sorting as a control. *C*, CaMKIIγB expression and (*D*) Ag restimulation of the sorted cells were performed as described in *A*.

P14*;γB** T cells expressed such a low level of *CaMKIIγB T287D* that a comparable amount of total CaMKIIγB was detected in P14*;γB** and P14 T cells. In contrast, T cells transduced with *CaMKIIγB T287D* expressed CaMKIIγB at levels that exceeded endogenous expression. In a second set of experiments designed to

address this issue, P14 T cells were transduced with *CaMKIIγB T287D* and then sorted for high or low expression based on the levels of Thy1.1 translated from the bicistronic message (Fig. 8*B*). The levels of Thy1.1 correlated with those of expressed CaMKIIγB (Fig. 8*C*). After 7 days in culture the sorted cells were harvested and tested for Ag-mediated proliferation. T cells expressing no transduced CaMKIIγB or low levels of active CaMKIIγB proliferated well in response to Ag peptide, whereas T cells expressing high levels of active CaMKIIγB did not respond at all (Fig. 8*D*). In subsequent sorted experiments, T cells expressing high levels of active CaMKIIγB were consistently unresponsive, while those expressing low levels of active CaMKIIγB were capable of responding to Ag restimulation but to a lesser extent than those expressing the vector control (data not shown). This observation suggested that the levels of active CaMKII in the sorted Thy1.1^{low} population were very close to the threshold of CaMKII activity that triggers T cell unresponsiveness. Taken together, these results indicated that the levels of CaMKII activation promote T cell proliferation or unresponsiveness upon Ag restimulation.

Active CaMKII-transduced T cells exhibit enhanced cytotoxic activity

To examine whether CD8 T cells transduced with active CaMKII exhibited altered effector function, we measured their cytotoxic activity against specific peptide-pulsed RMA-S targets. Activated P14 T cells were transduced with various viral preparations, purified for the expression of Thy1.1, and tested for CTL activity. As shown in Fig. 9, P14 T cells expressing vector, kinase-dead mutant, or wild-type CaMKIIβ'e exhibited similar levels of cytotoxic activity against gp33-pulsed RMA-S target cells. T cells expressing either active CaMKIIβ'e or CaMKIIγB killed gp33-pulsed RMA-S more efficiently. Although both were reproducibly greater than the controls, the effect of CaMKIIβ'e was greater than that of CaMKIIγB. In addition, the results indicate that active CaMKII expressed after an initial 24 h of activation enhances proliferation and cytotoxicity of CD8 T cells, but renders them almost completely unresponsive to restimulation with either Ag or PMA and ionomycin. In other experiments the very same T cells were shown
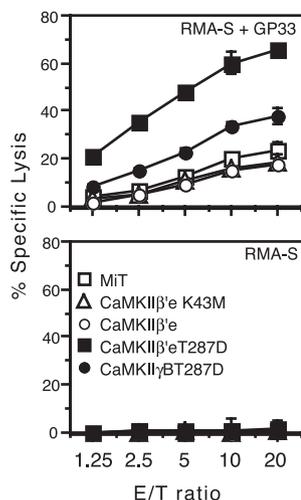

FIGURE 9. Enhanced CTL activity of P14 T cells expressing active CaMKII. The transduced T cells were purified with the Thy1.1 marker and determined for their CTL activity at 3 days after spin infection. The purities of the sorted cells were >90%. Specific lysis mediated by each population of T cells against RMA-S pulsed with or without gp33 was determined in a 6-h ^{51}Cr-release assay.

to exhibit unresponsiveness as measured by proliferation and enhanced cytotoxicity, and we note that this is somewhat reminiscent of "split tolerance" seen for CD8 T cells presented with Ag in the absence of costimulation (46).

Discussion

Previous work has shown that the expression of an active form of CaMKIIγB causes enhanced proliferation resulting in a long-term increase in the number of memory cells (22). We now show that another isoform of CaMKII, CaMKIIβ'e, is expressed in T cells, and it too can affect T cell physiology. The expression of an active form of either CaMKIIγB or CaMKIIβ'e after 1 day of T cell activation enhances initial proliferation and CTL activity, but induces a profound unresponsiveness to peptide restimulation. These data are consistent with a role for CaMKII in T cell differentiation.

CaMKIIβ has been characterized along with CaMKIIα for its roles in long-term potentiation, learning, and memory (47, 48). In contrast to the extensively characterized function of CaMKII in brain, the understanding of expression and function of this kinase family in lymphocytes is limited. A previous report showed a low-level expression of CaMKIIβ in human leukocytes (49); however, the study did not determine cellular subsets responsible for expression, nor the identity of the splice variants that were expressed. Gene array analysis shows that CaMKIIβ is mainly restricted to neuronal tissues, but it is also expressed at significant levels in other tissues including bone marrow and lymph nodes (50). We found that CaMKIIβ'e but not CaMKIIβ was expressed in both CD4 and CD8 T cells, and we have confirmed that T cells also express splice variants of CaMKIIγ and CaMKIIδ (our unpublished data). The neuronal form of CaMKIIβ, when overexpressed in T cells, was found concentrated in close proximity to the MTOC, and dispersed upon T cell activation (Fig. 2). In contrast, CaMKIIβ'e expressed in T cells does not appear to localize with F-actin, even under conditions of overexpression, nor does it associate with the MTOC. CaMKIIβ'e lacks two sequences found in the variable region, at least one of which was found to mediate cytoskeletal interactions (51). By fluorescence microscopy using YFP-tagged recombinant constructs, CaMKIIβ'e appears to be diffuse in the cytoplasm and unchanged in its localization as a consequence of Ag-mediated T cell activation.

The transduction of *CaMKIIβ'e T287D* into activated CD8 T cells caused a modest increase in proliferation that was not due to an enhanced secretion of IL-2, as T cells expressing CaMKIIβ'e T287D produced less IL-2 (Fig. 4*A*). We cannot rule out the production of other growth factors at low levels, but medium samples taken from these transduced cultures were not able to enhance the proliferation of untransduced, activated T cells (Fig. 4*B*). We did not find a similar effect on CD4 T cells; the transduction of CaMKIIβ'e into Ag-activated AND T cells did not result in enhanced proliferation (data not shown). The basis for this difference in CD4 and CD8 T cells is not understood, but it may relate to the differences in cytokine production or the differences in programmed proliferation (52–54).

A previous report has shown that activated T cells undergo IL-2-dependent and IL-2-independent cell cycle progression (55); so it is possible that CaMKIIβ'e may alter cell cycle progression in a cytokine-independent manner. CaMKII has also been shown to promote cell cycle progression in a calcium-dependent manner (56) and to associate with activators of cdk5 (57). Active CaMKIIβ'e or CaMKIIγB also enhanced killing activity (Fig. 9)

consistent with the ability of CaMKII to regulate the level of activation. Alternatively, since it has been shown that calcium signaling modulated perforin and FasL/Fas-mediated cytotoxic activity (58), the possibility exists that CaMKII may be directly involved in the regulation of CTL effector function.

The most striking effect of the expression of CaMKIIβ'e T287D or CaMKIIγB T287D in CD8 T cells was the induction of profound unresponsiveness (Figs. 5 and 7). We note that unresponsiveness induced in CD4 T cells stimulated in the absence of costimulation has been termed "anergy", and thus the use of this term has specific connotations concerning Ag-mediated self-tolerance. Unresponsiveness associated with CD8 T cells is less well-characterized (6), though as indicated below, there are at least two models of CD8 T cell unresponsiveness that appear to be dependent on Ag presentation (41, 44). The model we wish to investigate is that release of free calcium in cells activates calcium-calmodulin signaling through pathways that include calcineurin and CaMKII. If a cell otherwise receives a suboptimal signal, this is translated into unresponsiveness.

CaMKII-mediated unresponsiveness has some characteristics in common with anergy associated with CD4 T cells, but in other respects, it is quite different. Similar to CD4 T cell anergy, unresponsiveness can be reversed by prolonged culture in IL-2 (Fig. 5B) (43). Recent reports have shown that gene related to anergy in lymphocytes and other ubiquitin ligases are up-regulated in ionomycin-stimulated CD4 T cells (15, 59), whereas no up-regulation of gene related to anergy in lymphocytes was detected in CD8 T cells transduced with CaMKII (our unpublished data). How ionomycin-induced unresponsiveness relates to anergy defined using suboptimal Ag stimulation is presently unknown, and we suggest the possibility that ionomycin stimulation, rather than mimicking a process unique to anergy, amplifies a normal negative feedback mechanism of Ag-stimulated T cells. This process, regardless of its role in regulation, is crucially dependent on calcineurin activation since it is sensitive to cyclosporine A, but it is also possible that other calcium/calmodulin targets such as CaMKII play a role as well (14, 60). The transgenic expression of activated calcineurin actually confers hypersensitivity to T cells, and partially overcomes the requirement for the release of free calcium in T cell activation (61).

Unresponsiveness associated with CD8 T cells has been described for at least two different examples of exposure to Ag. AINR of CD8 T cells results from a lack of IL-2 signaling (44); alternatively, tolerance in vivo was achieved in response to suboptimal presentation of the HY Ag (41). CaMKII-induced unresponsiveness differs from AINR in that PMA and ionomycin restored proliferation and IL-2 production in AINR cells (44), whereas HY-specific T cells isolated from male mice produced IL-2, but did not proliferate in response to PMA and ionomycin (41). Another distinguishing characteristic of these two examples of unresponsiveness is rescue by the addition of excess IL-2. Exogenous IL-2 added during the Ag restimulation was reported to restore proliferation of AINR cells (45), but not male Ag-specific cells rendered tolerant in vivo. Likewise, we found that T cells transduced with active CaMKIIβ'e could not be restimulated with Ag in the presence of excess IL-2 (Fig. 6). Thus, constitutive expression of active CaMKIIβ'e induced CD8 T cell unresponsiveness that is distinct from AINR, but similar to the state of tolerance described by Tanchot et al. (41).

Previous studies on the role of CaMKIIγB yielded seemingly conflicting results. The transfection of Jurkat T cells with CaMKIIγB T287D inhibited IL-2 and IL-4 promoter activity, suggesting a role of CaMKIIγB in the induction of T cell anergy (20, 21). In contrast, we previously showed that there was an aug-

mented response and an increase in memory CD4 and CD8 T cells in CaMKIIγB T287D transgenic mice (22). The present report reconciles these data. We propose that CaMKIIβ/CaMKIIγ may play a role in either differentiation to memory cells or unresponsive cells depending on the level of expression and activation. As a precedent for this, calcium signaling in combination with other signaling pathways induced expression of NFAT and AP-1 leading to the activation of the T cells and hence generation of memory T cells, while unopposed calcium signaling up-regulated NFAT but not AP-1 resulting in unresponsiveness after Ag restimulation (14). In our previous studies, T cells from CaMKIIγB T287D transgenic mice expressed very low levels of active CaMKIIγB and were activated by TCR engagement and costimulation simultaneously. This led to the productive activation of the T cells, whereas higher levels of CaMKIIγB T287D in Jurkat T cells or the retrovirus-transduced T cells may evoke selective signal transduction. Consistent with this notion, we found that activated P14 T cells transduced with CaMKIIγB T287D were rendered unable to proliferate in response to Ag restimulation for days after stimulation (Fig. 7). We suggest that a strong calcium response in the absence of other requisite signals renders T cells unable to proliferate upon Ag restimulation, and that isoforms of CaMKII are, in part, responsible for this physiological state.

Whether this or other models of unresponsiveness, such as stimulation with ionomycin, accurately model the anergy associated with an absence of costimulation is presently unknown; however, an alternative possibility is that CaMKII is involved in the fate of activated, effector T cells. These T cells either acquire the characteristics of memory cells, remaining relatively quiescent but capable of rapid reactivation, or they become terminally differentiated cells, capable of effector function but destined never to participate in a secondary Ag-mediated response. The latter cells appear in large numbers under conditions of chronic Ag stimulation, and ultimately lose their ability to respond to Ag restimulation. We propose that CaMKII may be one factor in directing a T cell to these alternative fates. Depending on the strength of calcium stimulation, read as differences in the frequency or amplitude of calcium oscillations, or other factors related to T cell stimulation, CaMKII may promote differentiation to memory cells or terminal effector cells unresponsive to Ag restimulation. Further understanding of the role of CaMKII in T cell activation, survival and differentiation may require cell type-specific targeted deletions of CaMKIIβ and CaMKIIγ as well as identification of relevant downstream substrates for CaMKII activity in T cells.

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Disclosures

The authors have no financial conflict of interest.

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