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A Role for CaMKII in T Cell Memory

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Summary

In order to study the role of calcium/calmodulin kinase II (CaMKII) in T cells, we generated transgenic mice expressing CaMKIIγB* (T287D), a partially calcium-independent mutant of CaMKIIγB. In these mice, the size of the thymus was increased 1.5- to 2-fold, at least in part due to an increase in the lifespan of double-positive (DP) thymocytes. More importantly, there was an increase in the number of T cells in the secondary lymphoid organs that had acquired an antigen-dependent memory phenotype. These T cells were bonafide memory cells as assessed by a variety of criteria. In addition, T cells from wild-type mice acquired calcium-independent CaMKII activity after several rounds of antigen-stimulated division. We propose that CaMKII controls a distinct process of activation-induced cellular differentiation.

Introduction

The multifunctional calcium/calmodulin kinase II family consists of at least four different isoforms that mediate part of the cellular response to calcium in various tissues (Hanson and Schulman, 1992). The α and β isoforms of CaMKII are exclusively expressed in the brain, whereas the γ and δ isoforms of CaMKII are ubiquitously expressed (Tobimatsu and Fujisawa, 1989). In the brain, CaMKII function is intricately linked to the induction of LTP (Lisman and Goldring, 1988; Malinow et al., 1989; Deisseroth et al., 1995; Fukunaga et al., 1996) and spatial learning as shown by the analysis of mice that express a calcium-independent form of CaMKIIα or mice that are deficient in CaMKIIα (Silva et al., 1992a, 1992b; Soderling, 1993; Mayford et al., 1995, 1996; Giese et al., 1998). There is only a limited understanding of CaMKII function in other tissues (Nishimoto et al., 1991; Heist and Schulman, 1998).

CaMKII can reveal kinase activity in two ways. Upon calcium influx, calcium-calmodulin binds to CaMKII and induces high levels of kinase activity. Activated CaMKII phosphorylates various substrates including threonine 287 (for γ, T286 for α) in the CaMKII inhibitory domain (Miller and Kennedy, 1986; Braun and Schulman, 1995). Autophosphorylation of Thr-287 causes CaMKII subunits to acquire a high affinity for calmodulin in the absence of calcium, such that calmodulin can remain "trapped" on autophosphorylated CaMKII; this results in autonomous, or calcium-independent, CaMKII activity (Meyer et al., 1992).

CaMKII exists as a holoenzyme composed of 6–12 subunits, and the proportion of autophosphorylated subunits is determined by the frequency of calcium oscillations produced by influx through calcium-selective channels such as the postsynaptic N-methyl-D-aspartate (NMDA)-type glutamate receptors (Bennett et al., 1983; Kanaseki et al., 1991; Hanson and Schulman, 1992; De Koninck and Schulman, 1998). CaMKII can thus "store" the previous frequency of calcium oscillations in the form of phosphothreonines such that CaMKII is a biochemical decoder that contributes to at least one aspect of the frequency response function leading to LTP in hippocampal neurons. Conceptually, calcium-dependent activity reflects acute activation, whereas the level of autonomous CaMKII activity reflects the previous frequency of calcium oscillations.

In T cells, calcium is an important second messenger that contributes to the induction of proliferation, anergy, and cell death (Schwartz, 1996; Sloan-Lancaster and Allen, 1996; Alberola-Ila et al., 1997; McConkey and Orrenius, 1997; Berridge et al., 1998). It thus affects acute T cell activation and also long-term changes in antigen responsiveness. As a major downstream effector of calcium-calmodulin in T cells, CaMKII has the biochemical design to regulate activation, anergy, and memory. To determine the effect of CaMKIIγ on T cells, Nghiem et al. (1993) constructed a calcium-independent mutant of *CaMKIIγ-B* analogous to the *CaMKIIα* mutant used to study neuronal LTP. This mutant (*CaMKIIγB*) encodes a negatively charged aspartic acid substituted for the threonine 287 phosphorylation site and exhibited 37% autonomous activity. In Jurkat T cells, *CaMKIIγ-B** squelched IL-2 production (Nghiem et al., 1994), and this was interpreted as an indication that CaMKII may be a mediator of T cell anergy.

In order to understand how CaMKII regulates T cell physiology, and in particular the long-term generation of anergy or memory, we generated mice expressing the calcium-independent mutant of CaMKIIγB (CaMKIIγB*) in the T cell lineage. Furthermore, we developed an assay to specifically measure CaMKII activity in T cells. Herein, we report a previously unrecognized role for CaMKII in influencing immature T cell lifespan and T cell memory formation.

Results

Transgene Construction

A form of CaMKII found in human T cells is the B subtype of the gamma isoform, CaMKIIγB (Nghiem et al., 1993).

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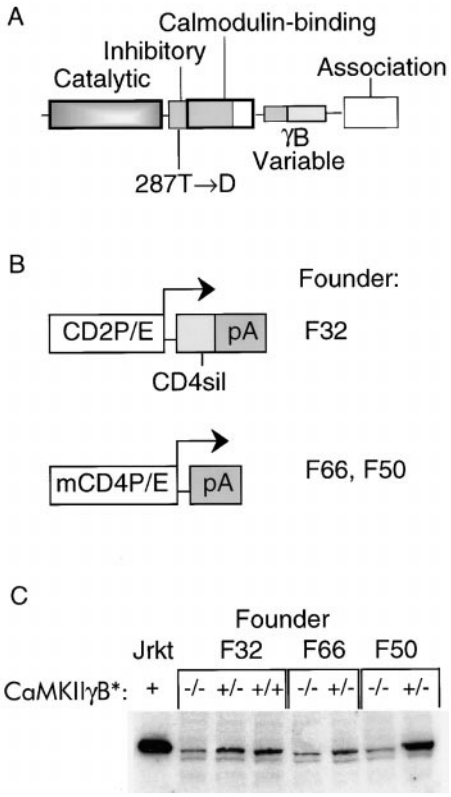

Figure 1. Trangenic Constructs and Transgene Expression

(A) Depiction of the human *CaMKIIγB* construct showing a point mutation in the autoinhibitory domain.

(B) Two expression cassettes were used to generate a total of six founders with variable expression. The CD4 silencer element is included in the human CD2-based expression cassette, though in this context it does not function (K. H. M., unpublished data).

(C) Western blot showing thymic expression of the transgene compared to endogenous CaMKII. As a control, Jurkat human T cell lymphoma cells expressing SV40 large T were transfected with the transgene. The transgene in founder 32 was made homozygous (+/+) to increase expression.

The partially calcium-independent mutant form of the human *CaMKIIγB* cDNA (*CaMKIIγB**) was subcloned into two expression cassettes, and six founders were obtained (Figures 1A and 1B). Transgenic line 32 bred for transgene homozygosity expressed roughly 3-fold the level of endogenous protein (Figure 1C). In this line, a combination of RT-PCR and Western analysis indicated transgene expression in all of the T lineages: CD4CD8 double-negative (DN), CD4CD8 double-positive (DP), and mature CD4 single-positive (CD4SP) and CD8SP T cells (data not shown). Much of the phenotypic analysis came from *CaMKIIγB*-32*, since it was the first one obtained. Line *CaMKIIγB*-50* expressed the transgene at about ten times the level of the endogenous message (Figure 1C), and preliminary analysis of this line fully corroborated the major observations made with *CaMKIIγB*-32* mice. For brevity, we will use *CaMKIIγB** to refer to *CaMKIIγB*-32$^{+/+}$* mice.

Thymic Phenotype

The most obvious effect of *CaMKIIγB** on thymic populations was a decrease in the proportion of SPs and an increase in the percentage of DPs (Figure 2A). A similar change in percentages was also seen in *CaMKIIγB*-50* mice (Figure 2, legend). The accummulated data from a large number of mice showed that the thymi from *CaMKIIγB** mice were about 1.5 to 2 times the size of control mice, and this increase was mostly in the progenitor DP population (Figure 2B). There was a slight decrease in CD4SPs but not in CD8SPs (data not shown). These differences in cell numbers remained consistent with age (3.5 weeks to 7.5 months), such that age-related thymic atrophy was normal in *CaMKIIγB** mice (data not shown). We tested the possibility that this phenotype could be explained by cell division; however, cell cycle analysis using 7-AAD (DNA content) and the incorporation of BrdU (DNA synthesis) showed that there was no difference in intrathymic proliferation (data not shown). We thus considered the alternative possibility that the *CaMKIIγB** gene affected cell survival.

The majority of DPs are not selected for further development, and kinetic labeling experiments showed that they undergo programmed cell death after a fixed lifespan of approximately 3.5 days (Huesmann et al., 1991; Shortman et al., 1991). We similarly measured the DP lifespan by the continuous administration of BrdU in vivo followed by an analysis of the incorporation of label into the nondividing DP population (Figure 2C). Whereas DPs from wild-type mice had a 3–3.5 day lifespan, DPs from *CaMKIIγB** mice showed a longer lifespan estimated to be 4–4.5 days. Consistent with an enhanced survival in the thymus, the DPs but not the SPs exhibited an enhanced survival in culture as measured by the number of surviving, annexin V-negative cells (Figure 2D).

In order to determine whether endogenous CaMKII activity regulates DP survival, we tested the effects of CaMKII inhibitors on thymocyte survival in organ culture. In separate experiments, we used KN62 and also the combination of KN93 and its inactive ortholog, KN92 (Tokumitsu et al., 1990; Sumi et al., 1991). The experiments consistently showed that the addition of CaMKII inhibitor caused a reduction in cell recovery in the DP population (Figure 2E). The recovery of SPs was also reduced and that could be due to decreased number of surviving DP precursors or a decrease in the survival of SPs themselves. The opposing effects of the transgene and the CaMKII inhibitors reveal that CaMKII regulates the survival and thus the lifespan of DP thymocytes.

The Phenotype of T Cells in the Peripheral Lymphoid Organs of *CaMKIIγB Mice**

The size of the spleen was increased in the *CaMKIIγB** mice, yet FACS analysis indicated no increase in the numbers of CD4 or CD8 splenocytes (Figure 3A). Preliminary experiments showed an increase in B cells in the spleen, and we do find expression of the transgene in B cells by RT-PCR. Analysis of the role of CaMKIIγ in B cells is ongoing.

Antigen activation causes CD4 T cells to increase the expression of CD44, decrease L-selectin (CD62L), and decrease CD45RB (Dutton et al., 1998). This phenotype remains even after the cells revert to quiescence, and it is thus used to characterize prior antigenic experience

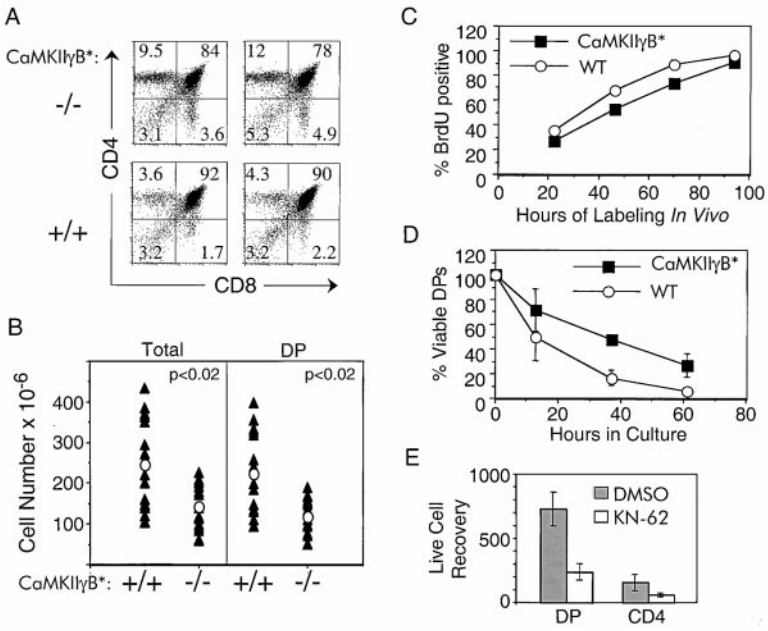

Figure 2. Thymic Phenotype of *CaMKIIγ-B** Mice

(A) Thymocytes from *CaMKIIγ-B** mice show an increase in the proportion of DP cells (two examples each).

(B) Number of thymocytes in 3.5- to 10.5-week-old mice. Absolute cell numbers for each thymic population were plotted compared to controls. Each triangle represents one mouse. A circle represents the mean. Two-tailed student p value is shown, where significant.

(C) In vivo lifespan of thymocytes. *CaMKIIγB** and control mice were continuously administered BrdU, and, at the times indicated, thymocytes were harvested and stained for percent incorporation of BrdU. Graph shows a summary of three experiments with 20 mice. Error bars are hidden by the symbols.

(D) The survival of thymocytes in culture. Thymocytes from *CaMKIIγ-B** and control mice were cultured in vitro, and, at the times indicated, cells were harvested and stained using Annexin V.

(E) The effect of a CaMKII inhibitor KN-62 on thymocyte viability in organ culture. Normal neonatal thymi were cultured in vitro with 10 μM KN62 or vehicle. Cell recovery was assessed after 3 days, and the scale indicates the total number of recovered viable cells for each lobe.

or "memory" T cells. Functionally, memory cells have been shown to produce a more rapid and varied cytokine response, and thus a secondary cellular immune response arises from an enhanced response by individual cells as well as an expansion of the antigen-reactive T cell clade. Previous work has shown that the proportion of T cells exhibiting a memory/activation phenotype increases with age, presumably due to immune responses to environmental antigens (Ernst et al., 1993; Sprent et al., 1997). In *CaMKIIγB** mice, we found a substantial increase in the percentage and number of T cells exhibiting a memory phenotype (Figures 3B and 3C). This enlarged population was found starting at 3 weeks of age and maintained such that *CaMKIIγB** mice consistently exhibited a 1.3- to 2-fold increase in the number of memory-phenotype cells. It was found in CD4 and CD8 subpopulations, in both the spleen and LN, and it was further exaggerated in *CaMKIIγB*-50* mice.

Conversion to the Memory Phenotype Is Antigen Specific

The physiological development of memory cells is, of course, antigen dependent, and this is supported by the observation that mice housed in germ-free facilities do not develop $CD45^{lo}$ T cells (Lee et al., 1990). Furthermore, in mice transgenic for T cell antigen receptor genes, T

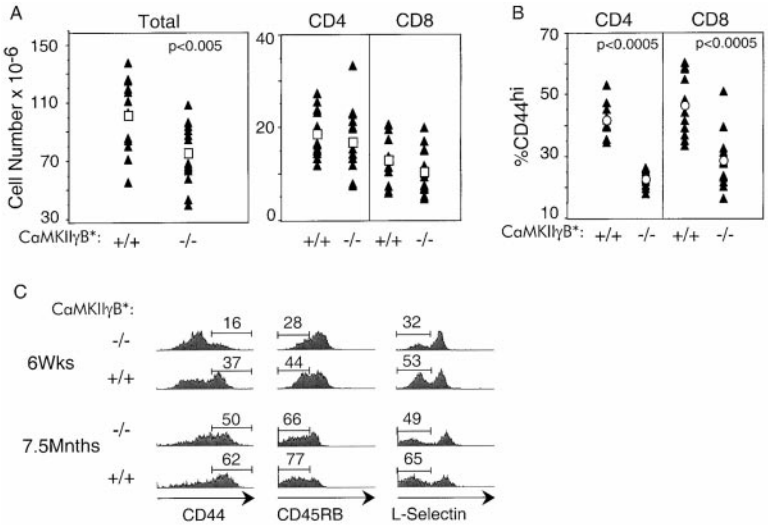

Figure 3. The Number of Memory T Cells in *CaMKIIγ-B** Mice Is Increased

Splenocytes from 4- to 10.5-week-old mice were counted and FACS analyzed. The number of cells was plotted for each of the indicated populations. Each triangle represents one mouse, and a square depicts the mean. Two-tailed student p value is shown, where significant.

(A) Total number of splenocytes and CD4 and CD8 T cells.

(B) The percentage of CD4 and CD8 T cells expressing high levels of CD44 as an indicator of activation/memory. The CD44 gate is shown in (C).

(C) The number of CD4 memory cells in *CaMKIIγ-B** and control mice at two different ages. The number of memory-phenotype cells was assessed by enumerating cells with high levels of CD44 and low levels of CD45RB and L-Selectin.

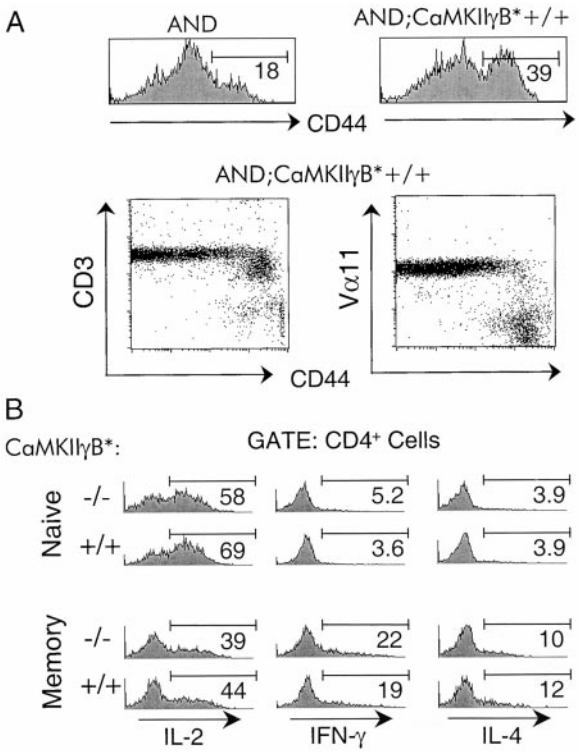

Figure 4. T Cells with a Memory Phenotype in *CaMKIIγB** Mice Are Functional

(A) The increase in CD44^{hi} cells is antigen dependent. Spleen cells from AND and AND;*CaMKIIγB** mice were analyzed for the expression of CD44 versus CD3 or transgenic Vα11.

(B) Memory-phenotype cells from *CaMKIIγB** mice secrete cytokines indicative of a memory response. Lymph node cells from *CaMKIIγB** and control mice were enriched for T memory cells (see the Experimental Procedures). IgG-depleted LN was used as a source of naive cells. After enrichment, cells were stimulated with 0.5 ng/ml PMA and 0.5 μM ionomycin for 14 hr.

cells expressing the transgenes do not convert to memory cells in the absence of antigen (Linton et al., 1996; Tanchot et al., 1997). AND transgenic mice possess TCRα and β chain transgenes that endow T cells with specificity for a peptide from pigeon cytochrome *c* bound to H-2E^k (Kaye et al., 1989). In order to examine the antigen dependence of memory cell formation in *CaMKIIγB** mice, we crossed *CaMKIIγB** with AND TCR transgenic mice to obtain mice that were genotypically *AND*$^{+/-}$;*CaMKIIγB**$^{+/+}$;H-2^b. They are unlikely to be exposed to pigeon cytochrome *c* in our mouse colony, and in any event, this peptide is not presented by H-2A^b, the only MHC class II molecule expressed by these mice. Similar to *CaMKIIγB** mice, the *AND*$^{+/-}$;*CaMKIIγB**$^{+/+}$;H-2^b mice had twice the percentage of CD44^{hi} cells when compared with *AND*$^{+/-}$;H-2^b mice (Figure 4A). The critical issue is whether the *CaMKIIγB** transgene is sufficient to convert cytochrome *c*-specific T cells to a memory phenotype or whether T cells that express the cytochrome *c*-specific TCR remain naive. Notably, although they expressed normal levels of the TCR-CD3 complex (Figure 4A, bottom left), the cells that expressed high levels of CD44 selectively did not express the transgenic α chain, Vα11 (Figure 4A, bottom right). This was also found for *CaMKIIγB**-50 mice. These data strongly suggest that enhanced conversion to a memory phenotype under the influence of the *CaMKIIγB** transgene requires the expression of an endogenous TCR, and we deduce that this event is thus antigen dependent.

Memory Cells Produce IL-4 and IFNγ

One hallmark of a memory cell population is the ability to secrete a wider diversity of cytokines (Picker et al., 1995; Dutton et al., 1998). We compared populations enriched for memory or naive cells from *CaMKIIγB** and control mice for their ability to synthesize IL-2, IL-4, and IFNγ. As depicted in Figure 4B, the naive cells produced IL-2 almost exclusively, whereas a larger proportion of memory produced IL-4 and IFNγ. Notably, there was no significant difference between cells from *CaMKIIγB** mice and wild-type mice in the ability to secrete cytokines. The CD8 memory-phenotype cells in *CaMKIIγB** and control mice exhibited a similar trend of cytokine secretion (data not shown). We deduce from these data that the memory-phenotype cells from *CaMKIIγB** mice have the functional capacity to secrete cytokines in an identical manner to memory cells from wild-type mice.

*CaMKIIγB** Mice Exhibit Enhanced Immune Responses

In order to understand the basis of the enhanced numbers of memory cells, we performed four types of experiments that measure the capacity of T cells to proliferate and survive both in vitro and in vivo. The first experiment measured the proliferative response of lymph node T cells from AND and AND;*CaMKIIγB** mice elicited by pigeon cytochrome c peptide. This is a response of naive cells since we showed that memory phenotype cells do not retain high levels of the cytochrome *c*-specific TCR (Figure 4A). As shown, there was no difference in the dose response curves, but the population of T cells from AND;*CaMKIIγB** mice synthesized DNA at a higher rate (Figure 5A). Identical results were obtained using *CaMKIIγB**-50 mice (data not shown).

In a second type of experiment, we inoculated mice with 10 μg of staphylococcal enterotoxin B (SEB), which activates T cells expressing Vβ8.1 and Vβ8.2, but not Vβ6 (White et al., 1989). The expansion and decline of these T cell populations was followed in the peripheral blood lymphocytes (Figure 5B). Initially, the number of Vβ8 T cells subsided, probably due to the fact that they are sequestered in the lymphatic system (Vasseur et al., 1999). This loss of Vβ8 T cells preceded a superantigen-mediated expansion followed by a decline shown to be accompanied by apoptosis (Kawabe and Ochi, 1991; MacDonald et al., 1991; Webb et al., 1994). This was found with identical kinetics for CaMKII and wild-type littermate mice, with the exception that we consistently found an increase in the magnitude of the expansion in vivo (Figure 5B) (see the Discussion). In the same experiments, nonresponding Vβ6 T cells were monitored and found to undergo no appreciable changes (data not shown).

We next tested the ability of the *CaMKIIγB** mice to respond to antigen after a previous immunization. A

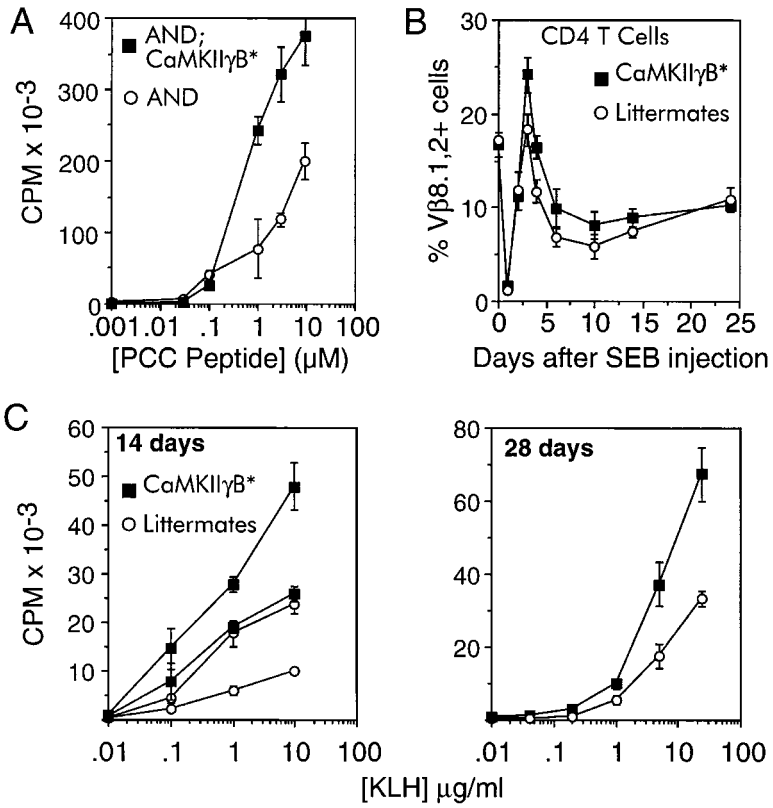

Figure 5. Enhanced Immune Responses in *CaMKIIγ-B** Mice

(A) Enhanced response to PCC T cell from *CaMKIIγ-B** mice. Lymph node cells from AND;*CaMKIIγ-B** and AND mice were cultured for 3 days and pulsed with 1 μCi of ^{3}H-thymidine for the last 20 hr.

(B) Vβ8$^+$ T cell expansion and deletion in vivo was induced by SEB. *CaMKIIγ-B*-50* and wild-type littermates (both H-2^{bxd}) were inoculated with 10 μg of SEB i.v., and, at the times indicated, mice were bled. The number of Vβ8$^+$ and Vβ6$^+$ in blood, counted as a percentage of the total CD4$^+$ T cells, was determined. Data are mean and standard deviation of values from five mice for each group.

(C) *CaMKIIγ-B** and control mice were immunized with 100 μg of KLH-DNP and complete Freunds adjuvant in the hind footpad. At the indicated times postimmunization, draining LNs were harvested and stimulated in vitro with KLH and syngeneic APCs for 3 days.

functional CD4 memory response was assayed by immunization with KLH-DNP in complete Freund's adjuvant followed by restimulation in culture (Figure 5C). The results showed that the *CaMKIIγB** mice had an enhanced in vitro recall response to KLH. As seen in the previous experiment, the dose response curves were unchanged in that the cells responded to the same range of antigen concentrations.

In order to determine the mechanism by which CaMKII enhances proliferation, we analyzed the kinetics of progression through the cell cycle and the proportion of cells that undergo apoptosis. Lymph node cells from AND and AND;*CaMKIIγB** mice (H-2^b) were stimulated in culture with antigen-presenting cells (H-2^{bxk}) and PCC for 1–3 days. The cells were labeled with the fluorescent dye, CFSE, at the initiation of culture, and the number of cell divisions was followed by the dilution of fluorescence. Cells were gated for CD4 and Vα11 and the populations analyzed for the number of cell divisions (Figure 6). Starting at day 2, the population of T cells (CD4; Vα11$^+$) from AND;*CaMKIIγB** mice included cells that had undergone at least one extra division relative to cells from AND controls. For example, at day 3, AND T cells stimulated with 1 μM PCC included a significant number of cells that had undergone 0, 1, 2, or 3 rounds of division, with a barely detectable population that had undergone 4 rounds of division. In contrast, T cells from AND;*CaMKIIγB** mice included cells that had gone through 0, 1, 2, 3, 4, or 5 rounds of division. This result suggests that CaMKII autonomous activity can either shorten the cell cycle or increase cell survival. In order to distinguish between these possibilities, we examined the number of cells undergoing apoptosis by simultaneously labeling with 7AAD (Philpott et al., 1996) (Figure 6). The proportion of apoptotic cells is plotted as a function of the cell divisions for each condition. The results show that, especially by day 3, there was a significant decrease in the proportion of apoptotic cells in the cultures from AND;*CaMKIIγB** mice. This is consistent with a mechanism of action whereby the autonomous form of CaMKIIγB* provides a survival signal that allows cells to choose a memory fate as opposed to activation-induced cell death. Although this explanation accounts for at least some of the activity of CaMKIIγB*, we cannot yet exclude the possiblity that there is also an effect on the progression of the cell cycle.

CaM Kinase II Activity in Activated, Dividing T Cells

To specifically measure endogenous CaMKIIγ activity, we immunoprecipitated (IP) CaMKIIγ before performing the kinase reaction. We found an increase in activity from thymocytes isolated from *CaMKIIγB** versus control mice, as well as in *CaMKIIγ-B**-transfected versus control Jurkat cells (Figure 7A). This increase was seen in both autonomous and calcium-dependent activity, confirming the specificity of the assay. Furthermore, this result provided evidence that the *CaMKIIγB** transgene encoded the expected biochemical activity. In normal unstimulated thymocytes, we could not detect autonomous activity above background, whereas thymocytes from *CaMKIIγB** mice showed 10%–20% autonomous activity. The calcium-dependent activity in *CaMKIIγB** mice was 2- to 3-fold higher than in wild-type mice,

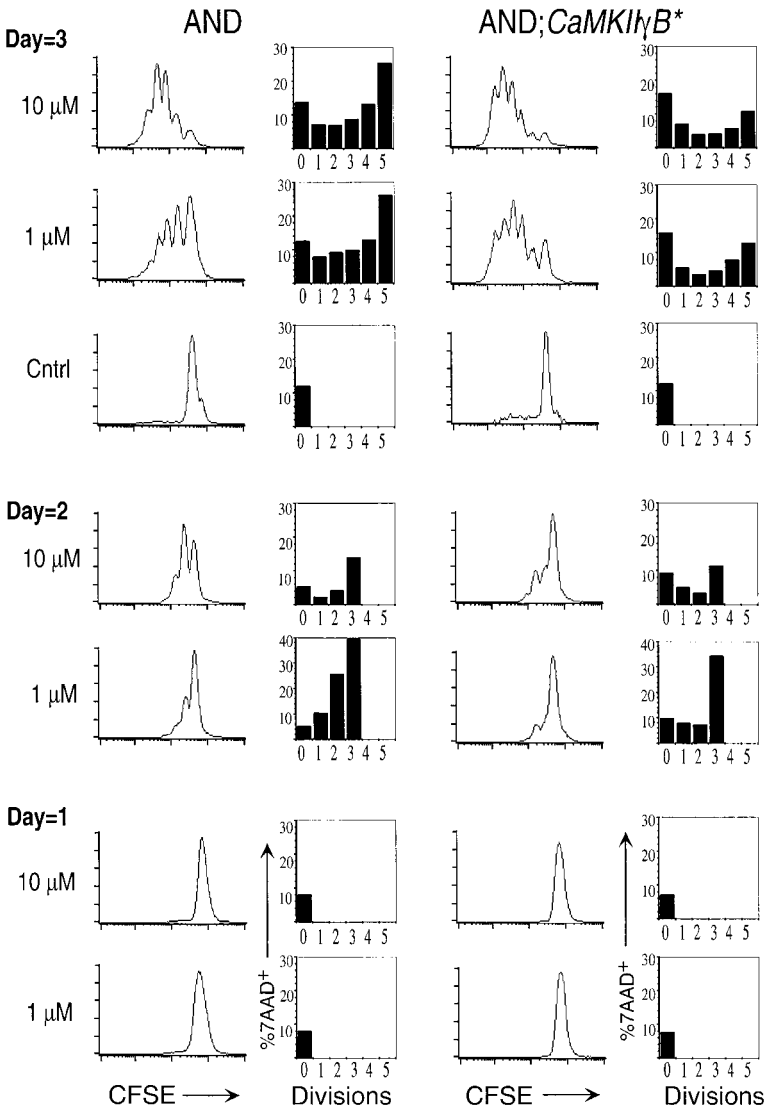

Figure 6. T Cells from CaMKII Mice Undergo More Rounds of Division and Less Activation-Induced Cell Death

Lymph node cells from AND;*CaMKIIγ-B*-50* and AND littermates were labeled with CFSE and stimulated in vitro with 0, 1, or 10 μM of moth cytochrome c peptide 88–103 (MCC). Each day, $CD4^+V\alpha11^+$ cells were gated for CFSE fluorescence and for the percentage of cells that stained with 7AAD.

consistent with the level of overexpressed protein as detected by Western blots.

The phenotypic results suggest that CaMKIIγ contributes to the formation of a memory T cell response. This could come about in one of two ways: CaMKII may regulate the formation of memory cells versus activation-induced apoptosis; alternatively, CaMKII could provide an epigenetic mechanism for maintaining T cell memory. To begin to distinguish between these two possibilities, we measured CaMKII activity in wild-type memory versus naive cells. We used two methods to enrich for memory and naive cells in order to avoid potential artifacts introduced by antibody-mediated cellular manipulations. We also added another control for specificity by carrying out the reactions in the presence and absence of a CaMKII peptide inhibitor (CKI) (Smith et al., 1992). Since the level of autonomous activity was low, we used 1/10 the amount of lysate for the calcium-dependent versus the autonomous reaction. The results revealed no significant difference in the calcium-dependent or autonomous activity in naive versus memory populations (Figure 7B).

Since we could not detect increased CaMKII activity in memory versus naive cells, we reasoned that CaMKII activity must be important for the induction rather than maintenance of T cell memory. We tested the hypothesis that CaMKII activity increases upon activation and cell division by stimulating purified AND TCR transgenic cells in vitro with pigeon cytochrome *c* peptide and antigen-presenting cells from B10.A mice. Cells were cultured in the presence of antigen and antigen-presenting cells and supplemented with 20 U/ml rIL-2 after 48 hr. Cell lysates were generated from cells stimulated for different lengths of time and assayed for CaMKII activity compared to unstimulated cells. Cells were also labeled with CFSE at time 0 and analyzed by flow cytometry to determine the distribution of cell divisions (Figure 7C). The level of autonomous (calcium-independent) CaMKII activity increased at days 4 and 5 but not at day 2, suggesting that autonomous CaMKII activity increases not simply in response to activation signals but in concert with antigen- and IL-2-mediated cell division (Figure 7D). The magnitude of the increase by densitometry was approximately 1.4-fold over the background at time zero.

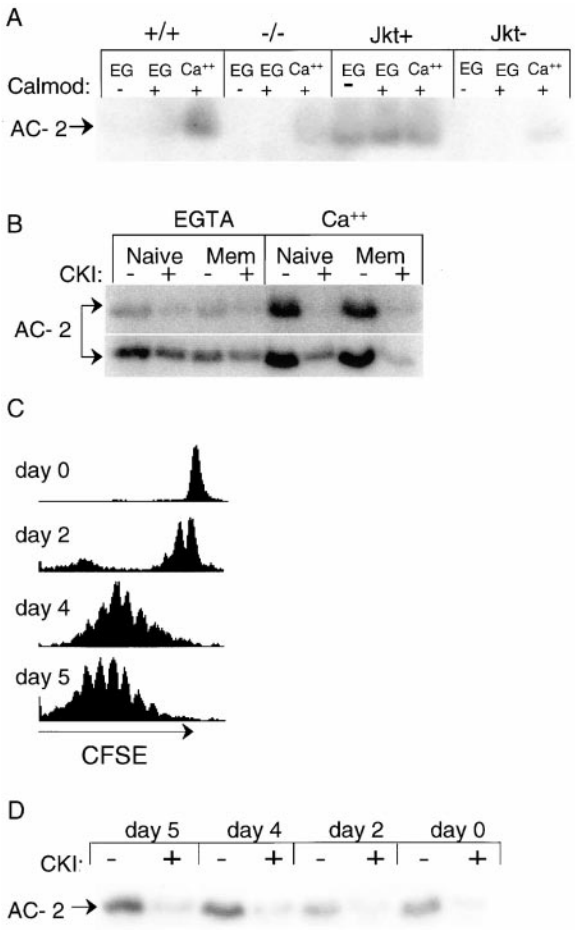

Figure 7. CaMKII Kinase Activity in T Cells from Wild-Type Mice

(A) CaMKIIγ activity in thymocytes and Jurkat T cells. Lysates were isolated from thymocytes explanted from *CaMKIIγ-B** and control mice as well as Jurkat lymphoma cells transfected with *CaMKIIγ-B** or empty vector.

(B) Naive and memory cells from wild-type mice have equivalent basal calcium-independent CaMKII activity. Naive and memory cells were purified by FACS sorting (top) or magnetic bead negative selection (bottom). FACS resulted in 98% purity in memory and naive populations. Magnetic bead depletion resulted in enriched populations: naive cells, 84% $CD44^{lo}$, 11% $CD44^{hi}$; memory cells, 33% $CD44^{lo}$ and 51% $CD44^{hi}$ T cells. Where indicated, 20 μM of CaMKII inhibitory peptide (CKI) was added to the kinase reaction.

(C) Cell division of antigen-activated cells. Transgenic AND CD4 LN cells were labeled with CFSE and stimulated in vitro by mitomycin c-treated APC and 1 μM PCC peptide. Twenty U/ml IL-2 was added on day 2. At the times indicated, cells were analyzed by FACS for the intensity of CFSE fluorescence.

(D) CaMKII autonomous activity was increased during late-stage antigen activation. Non-CFSE-labeled transgenic AND CD4 LN cells were activated in parallel with cells from (C), and, at the times indicated, cells were harvested for the IP kinase assay. Where indicated, 50 μM CKI was added to the kinase reaction.

Discussion

Memory T Cells

There is no reason a priori that memory in the nervous system shares more than an etymological similarity to memory in the immune system. We were thus somewhat surprised to find that a major biochemical decoder of previous activation in the nervous system may play an analogous role in lymphocytes. Yet, CaMKII is ubiquitously expressed, and its tailored biochemical characteristics were presumably selected to function in a variety of cell types. Genetic and biochemical processes are often used and coopted during evolution, so, in retrospect, the notion that there are distant parallels between the memory of experience and the memory of antigen experience may have a basis in fact. In this report, we document that a point mutation in *CaMKIIγB* causes two readily apparent effects on the physiology of the immune system. Most notably, CaMKIIγB* enhances the antigen-dependent formation of T cell memory. This reverse genetics approach was reinforced by the observation that the activation and induction of cell division in primary cultures of T cells causes an increase in endogenous CaMKIIγ activity. A second observation, that may or may not be related, is that CaMKIIγB* lengthened the DP thymocyte lifespan.

We deduce that the memory phenotype of cells from *CaMKIIγB** mice, as defined by the expression of CD44, CD62L, and CD45RB cell surface markers, is indicative of memory T cells able to mediate a secondary immune response. First, the conversion to the memory-phenotype increased with age, similar to that which is seen in wild-type mice. Second, the increase in memory-phenotype cells was specific for cells with the potential to recognize environmental antigens, i.e., cells with a transgenic TCR that expressed CaMKIIγ-B* remained naive. Third, memory-phenotype cells from *CaMKIIγB** mice expressed an expanded range of cytokines after stimulation that was indistinguishable from wild-type memory cells (Cho et al., 1999). Fourth, T cells from *CaMKIIγB** mice exhibit an enhanced recall response to antigen. One issue concerns the long-term survival of peripheral blood T cells stimulated by SEB. Based on the steady-state increases in memory cells in the peripheral lymphoid organs, we might have expected the Vβ8 T cells to persist at higher levels in *CaMKIIγB** mice. That fact that SEB causes an initial increase, but no enhanced persistence, is probably related to the observation that superantigen activation leads not to memory but rather to clonal exhaustion and unresponsiveness (Rammensee et al., 1989; Rellahan et al., 1990; McCormack et al., 1993; Webb et al., 1994). Indeed, if the *CaMKIIγB** transgene did lead to memory under these circumstances we would worry that the effect was not physiologically significant. In every assay, the effect of *CaMKIIγB** was to enhance a natural process of proliferation and memory formation.

We believe that there are two possibilities to explain an increase in the number of memory cells. One relates to the fate of antigen-activated T cells. Such cells either undergo activation-induced cell death or differentiate to form memory cells, and it is possible that CaMKIIγ regulates this fate decision. A second possibility is that the lifespan of the memory cell could be increased; however, this possibility is not tenable given the recent work showing that memory cells have an indefinite lifespan even in the absence of antigen (Sprent et al., 1997; Murali-Krishna et al., 1999; Swain et al., 1999). We favor instead the idea that CaMKII regulates the differentiation into memory cells but not the maintenance of the memory phenotype. In support of this interpretation, we

found there to be an increased antigen or superantigen-induced expansion of T cell in culture or in vivo. Moreover, at least part of this increase can be explained by a decrease in activation-induced cell death (Figure 6). The notion is that memory cells arise from dividing effector cells and the level of CaMKII autonomous activity is an important regulator of survival and differentiation at this critical step. Remarkably, this is consistent with the role of CaMKII in the induction but not the maintenance of LTP (Malinow et al., 1989; Otmakhov et al., 1997).

In the formation of learning and memory in the brain, CaMKII acts as a decoder of the frequency of calcium oscillations (De Koninck and Schulman, 1998). Higher frequencies result in LTP, and lower frequencies result in long-term depression. This decoding function, and memory itself, is critically dependent on the establishment of autonomous CaMKII kinase activity through the phosphorylation of threonine 286 (Giese et al., 1998). Is it possible that CaMKII in T cells is regulated in the similar fashion? A body of work has shown that calcium oscillations occur in nonexcitable cells, and both frequency and amplitude can be determined by the characteristics of agonist-receptor interactions (Thomas et al., 1996). In particular, ligation of the TCR causes calcium oscillations in T cells, and the oscillatory frequency can be the determining factor in activation of transcription factors such as NF-AT and NFκB (Dolmetsch et al., 1997, 1998). Furthermore, recent evidence indicates that memory T cells arise from activated T cells that have undergone numerous rounds of activation-induced cell division (Opferman et al., 1999). We suggest that the duration of signaling through the TCR and other factors, such as coreceptor stimulation, may affect the frequency and perhaps the amplitude of calcium oscillations. This, in turn, could determine the level of autonomous CaMKII activity and the probability of survival as a memory cell.

Although we wish to draw an analogy between survival and memory in lymphocytes and LTP in neurons, there are most certainly important differences. Intracellular calcium oscillations in lymphocytes result from the release of calcium from intracellular stores and the activation of store-operated calcium (CRAC) channels in the plasma membrane (Zweifach and Lewis, 1993). In contrast, calcium oscillations in postsynaptic cells result from an entirely different mechanism that involves calcium influx through NMDA receptors (Artola and Singer, 1993). In addition, lymphocyte memory involves survival of proliferating cells in the face of activation-induced cell death, whereas LTP occurs in a nondividing neuronal population. We imagine that CaMKII is but one processor of cell-type specific input and functions to initiate differentiation programs that are entirely divergent in lymphocytes and neurons.

We note that survival is not sufficient for enhanced memory T cell formation. In mice expressing a Bcl-2 transgene in the T cell lineage, activation-induced cell death was decreased, but there was no observed increase in the number of memory cells (Strasser et al., 1991; Petschner et al., 1998). Yet, whereas Bcl-2 is not sufficient to enhance memory cell formation, it is possible that it is a downstream target of CaMKII.

Thymocyte Lifespan

An extravagant aspect of T cell development is the massive cell death that occurs as a natural part of thymic selection. Rather than die continuously with a constant probability, thymocytes have a fixed lifespan of approximately 3.5 days (Huesmann et al., 1991; Shortman et al., 1991), and we have found that this fixed lifespan is manifested in thymocyte survival in vitro as well (J. D. B., unpublished data). Thus, there must be a mechanism to keep track of time in this nondividing population. Since *CaMKIIγB** mice displayed an extended lifespan both in vivo and in vitro, the calcium-independent form of CaMKIIγ thus appears to regulate the timing of programmed cell death. This may be a key to its function, both in the thymus and the generation of memory T cells.

Again, there are similarities, but also important differences in comparing the thymic phenotypes of *Bcl-2* and *CaMKIIγB* transgenic mice. Both DP and SP thymocytes from Bcl-2 transgenic mice exhibit an increased lifespan measured in vitro (Sentman et al., 1991; Strasser et al., 1991), and we have found that DP thymocytes from Bcl-2 transgenic mice label very slowly with an estimated in vivo lifespan of more than 8 days (J. D. B. and S. M. H., unpublished data). Despite this profound effect on survival, Bcl-2 mice do not have an increase in total thymocytes but do show a greater number of SPs and a skewed selection toward the CD8 lineage (Linette et al., 1994; Tao et al., 1994). We conclude that, separate from the survival effects of Bcl-2, CaMKII plays a part in the strict control of DP lifespan. In this regard, it will be worthwhile to dissect how CaMKII may link up to the mechanisms that measure time.

Experimental Procedures

Animals

All mice were bred and maintained in the animal facility at the University of California, San Diego. AND T cell antigen receptor transgenic mice were previously described (Kaye et al., 1989). *CaMKIIγ-B*-32* transgenic mice were generated at the Scripps Research Institute by Jenny Price. *CaMKIIγ-B*-50* transgenic mice were generated by the UCSD Transgenic Core by Michelle Paulus. Inbred mice were obtained from Jackson Laboratory.

Cell Culture, NTOC, and Transfection

Thymus, LN, and spleen cells were harvested and maintained in RPMI media as previously described (Sharp et al., 1997). Two rounds of B cell depletion were carried out using sheep anti-mouse IgM from Dynal as per manufacturer's instructions. Memory cells were enriched by depleting for B cells and MEL-14 (L-selectin) positive cells. Jurkat cells were transfected as described with an efficiency of 5%–30% (Jacinto et al., 1998). For NTOC, neonatal thymus organs were removed from 1-day-old pups and cultured as described (Sharp et al., 1997).

For recall responses, mice were immunized with 100 μg KLH in complete Freund's adjuvant. Cells were cultured with APC and KLH for 72 hr and pulsed with 1 μCi ^{3}H-thymidine for the last 20 hr of culture. AND proliferation cultures were done as previously described (Sharp et al., 1997). KLH-DNP and KLH were from Calbiochem, Inc. (La Jolla, CA). Pigeon cytochrome c peptide 88–104 (PCC) and moth cytochrome c peptide 88–103 (MCC) were synthesized at the UCSD peptide facility. Complete Freund's Adjuvant (CFA) was from Sigma Chemical Co.

Plasmids and Transgenesis

SRαCaMKIIγ-B* was obtained from Colleen Sheridan (Nghiem et al., 1994). The insert along with the CD4 silencer was cloned into

the XhoI site of pTEX (Zhumabekov et al., 1995) (details available upon request). The expression cassette was liberated from the plasmid for microinjection. Eight founders were obtained of which two expressed mRNA and one was maintained (*CaMKIIγ-B*-32*). By Southern blot analysis, we estimate that there are one to two copies integrated for this line. For generation of *CaMKIIγ-B*-50* mice, *CaMKIIγ-B** was cloned into LL428 (N. Killeen, UCSF). LL428 is derived from construct "i" in Sawada et al. (1994). The expression cassette was separated from the plasmid for microinjection. Five founders were obtained, of which two expressed mRNA.

Primers

One set of primers was used for detection of the transgene in genomic DNA and in cDNA (5′ primer, 5-ATGATGCATCGTCAGGAGAA-3; and 3′ primer, 5-ACTGTTTTTGTTGTTGCTCTG-3). These primers did not cross-react with nontransgenic genomic DNA or endogenous CaMKII cDNA, since the mutated codon was included at the 3′ end of the 5′ primer. Primers to β-actin were used as controls.

Flow Cytometry

Antibodies to CD4 and CD8 and recombinant human Annexin V-FITC were from Caltag. Antibodies to CD5, CD62L, CD45RB, CD69, CD44, CD25, CD3, IL-2, IL-4, and IFNγ were from Pharmingen. Antibody to BrdU was from Becton Dickinson. BrdU staining was done as described (Tough and Sprent, 1994). Staining for apoptotic cells using 7-amino actinomycin D (7AAD) was performed as described (Philpott et al., 1996), and the method for CFSE and intracellular cytokine staining was taken from Bird et al. (1998).

Western Blots

Anti-CaMKIIγ was from Santa Cruz Bio. (sc-1541). Cells were lysed in high-salt lysis buffer (20 mM Tris [pH 7.6], 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 5 mM beta-glycerol phosphate, 0.5% NP-40, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 200 μM PMSF, 1 mM benzamidine, 10 μM NaF, 30 nM okadaic acid [OA], and 200 μM $NaVO_4$). Ten to fifty micrograms total protein was separated by SDS-PAGE and transferred to nitrocellulose. Visualization was by ECL (Amersham). The membrane was blocked in hTBS-T (20 mM Tris [pH 8], 500 mM NaCl, and 0.1% Tween 20) + 5% milk. Primary antibody was used at 1:1000 in hTBS-T for 1 hr at room temperature. Membrane was washed 4× in hTBS-T, and secondary incubation was for 30 min in hTBS-T with 1:2000 rabbit anti-goat HRP (Southern Biotech).

Kinase Assays

Cells (3–10 × 10^6) were lysed for 5 min on ice in 50–100 μl high-salt lysis buffer. The lysate was precleared with 30 μl protein G Fast Flow (Pharmacia, 50% mix) for 5–30 min. Protein concentration was determined by Bio-Rad assay. Thirty to one hundred micrograms lysate was bound in high-salt lysis buffer with 5 μg anti-CaMKIIγ and 30 μl protein G overnight. Lysate was spun and beads washed 4× with high-salt lysis buffer (no protease inhibitors) and 1× with kinase buffer (50 mM PIPES [pH 7], 10 mM $MgCl_2$). The kinase reaction was started by the addition of 15 μl CK kinase reaction buffer (50 mM PIPES [pH 7], 10 mM $MgCl_2$ + 10 μg/ml BSA, 1 μm DTT, 10 μl cold ATP, 5 μCi γ-P^{32}-ATP, and 10 μM AC-2 [Calbiochem]) with combinations of 10 μg/ml human brain calmodulin (Calbiochem), 4 mM EGTA, or 2 mM $CaCl_2$, and incubation was at 30°C. Reaction was stopped by addition of 8 μl 6× sample buffer after 5–10 min or after 80 s for EGTA or Ca^{+2} conditions, respectively. Kinase reactions were run on a 20% 2× gel for resolution of AC-2.

SEB Responses In Vivo

CaMKIIγB-50* transgenic mice were crossed with B10.D2 mice to produce *CaMKIIγB*-50*;H-2^{bxd} and wild-type H-2^{bxd} offspring. These mice express H-2E molecules that allow them to respond to SEB at low doses. Ten micrograms of SEB was injected intraperitoneally in PBS. Blood from each mouse was collected at different time points, and white blood cells were stained with FITC anti-Vβ8 or anti-Vβ6 (Pharmingen), PE anti-CD4 (Pharmingen), and Tricolor anti-CD8 (Caltag).

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