Calmodulin-dependent Protein Kinase IV Regulates Hematopoietic Stem Cell Maintenance

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The hematopoietic stem cell (HSC) gives rise to all mature, terminally differentiated cells of the blood. Here we show that calmodulin-dependent protein kinase IV (CaMKIV) is present in c-Kit+ Sca-1 Lin−/low hematopoietic progenitor cells (KLS cells) and that its absence results in hematopoietic failure, characterized by a diminished KLS cell population and by an inability of these cells to reconstitute blood cells upon serial transplantation. KLS cell failure in the absence of CaMKIV is correlated with increased apoptosis and proliferation of these cells in vivo and in vitro. In turn, these cell biological defects are correlated with decreases in CREB-serine 133 phosphorylation as well as in CREB-binding protein (CBP) and Bcl-2 levels. Re-expression of CaMKIV in Camk4−/− KLS cells results in the rescue of the proliferation defects in vitro as well as in the restoration of CBP and Bcl-2 to wild type levels. These studies show that CaMKIV is a regulator of HSC homeostasis and suggest that its effects may be in part mediated via regulation of CBP and Bcl-2.

The intracellular Ca2+ receptor calmodulin (CaM) and its downstream CaM-dependent protein kinases (CaMK) I, II, and IV connect transient increases in intracellular Ca2+ with physiological processes such as proliferation, development, and differentiation. CaMKIV is a multifunctional serine/threonine (Ser/Thr) protein kinase found predominantly in cells of the brain, testis, thymus, and ovary as well as in mature T cells and neutrophils (1–7). This predominantly nuclear protein kinase regulates transcription mediated by several transcriptional activators including CREB (8), CBP (9, 10), MEF2 (11), RORα (12), and COUP-TF (12), in response to transient increases in intracellular Ca2+.

To evaluate the physiological roles of CaMKIV, two independent C57BL/6/J × 129Sv lines of Camk4−/− mice were generated using different targeting strategies (7, 13). Both lines of Camk4−/− mice revealed deficits in brain (4, 13, 14) and T cell function (1). Furthermore, targeted expression of a kinase-inactive CaMKIV in mice results in defective thymocyte survival and activation (2). Although the precise cascade of events in which CaMKIV participates remains enigmatic, neurons (13, 14) and memory T cells (1) for Camk4 show a marked decrease in CREB Ser133 phosphorylation (phospho-CREB), indicating that CREB-mediated transcription may contribute to the observed phenotypes. In addition, CaMKIV has been shown to phosphorylate CBP at Ser301, thereby enhancing CREB-CBP-mediated transcription (10). Such findings have led to the hypothesis that a CaMK cascade, of which CaMKIV is a component, is a part of the pathway by which Ca2+ regulates transcription mediated by CREB and CBP (15).

In this report, we investigated whether CaMKIV is involved in early hematopoietic development and found that the absence of CaMKIV results in a reduction in the number of c-Kit+ Sca-1 Lin−/low cells (KLS cells), a cell population that includes long-term and short-term hematopoietic stem cells as well as other multipotent progenitor cells (16). Specifically, we found that the Camk4 gene is expressed in KLS cells and that CaMKIV is required for KLS cells to repopulate the bone marrow in transplantation assays. Furthermore, Camk4−/− KLS cells display enhanced proliferation as well as increased apoptosis, in vivo and in vitro, compared with wild type (WT) cells and have decreased levels of phospho-CREB (pCREB), CBP, Bcl-2 mRNA and Bcl-2 protein. Re-expression of CaMKIV in Camk4−/− KLS cells restores Bcl-2 and CBP levels and rescues the proliferation defects. Thus, our data reveal a novel role for CaMKIV in the maintenance of hematopoietic homeostasis and suggest that this role involves suppression of inappropriate KLS cell proliferation.

EXPERIMENTAL PROCEDURES

Mouse Strains—The Camk4−/− mouse was generated using a CaMKIV target vector construct that deletes the first two exons of Camk4 and the two known transcription initiation sites (7). Genotyping was performed as described previously (7). In the mixed genetic background of C57BL/6J × 129Sv, ~50% of the Camk4−/− pups showed growth retardation and died within the first 3 weeks of postnatal life. The remaining mice grew to adulthood but were infertile. Because these severe defects did not occur in the other line of Camk4−/− mice generated by Ho et al. (13), we initiated a breeding program for nine generations to stabilize the genetic background. This resulted in the loss of the fertility and premature death phenotypes but the brain and T cell phenotypes were maintained. All mice used in the present study were fertile, grossly asymmetrical and lived a normal life span. All animals were housed and maintained in the Levine Science Research Center Animal Facility located at Duke University under a 12-h light, 12-h dark cycle. Food and water were provided ad libitum, and all care was given in compliance with National Institutes of Health and institutional guidelines on the use of laboratory and experimental animals.
Bone Marrow Histology—Femurs isolated from 8-week-old mice were fixed in fresh 4% paraformaldehyde for 48 h, washed in 70% ethanol, and decalcified for 72 h. Glycol methacrylate infiltration and embedding were performed using JB-4 embedding kit (Polysciences, Warrington, PA). Two-μm sections were prepared and stained with hematoxylin and eosin.

White Blood Cell Differentials—Blood was extracted for analysis by cardiac puncture. Blood cell counts were performed using automated analysis on a System 9000 Automated Cell Counter (Serono-Baker Diagnostics, Allentown, PA).

Colony Forming Assays—Colony forming assays were performed by plating 1 × 10^4 unfractionated bone marrow cells in quadruplicate on Methocult methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) and grown at 37 °C in 5% CO2. The evaluation of colony forming units was performed after 2 weeks in culture as per manufacturer’s protocol.

Isolation of Hematopoietic Stem Cells—Isolation of HSCs from bone marrow cells was performed using a FACSVantage (BD Biosciences) as described (16). In particular, HSCs were sorted based on positive expression of c-Kit and Sca-1 (c-Kit+/Sca-1+) and low/negative expression of the lineage markers (Lin−/low).

Stem Cell Transplantation—Bone marrow transplants were performed using the congenic strain B6.SJL-Ptprc−/− Pep3b/Boy (CD45.1, Jackson ImmunoResearch Laboratories, West Grove, PA) as the recipient. Recipient mice, older than 10 weeks of age, were irradiated by exposing them to a single dose of 9.5 Gy 137Cs source. The following day, c-Kit+, Sca-1+, Lin−/low hematopoietic stem cells (KLS cells) were isolated from 3-week-old wild type and Camk4−/− (both CD45.2) donor mice. About 1000 sorted KLS cells from one donor were injected into the retro-orbital sinus of five to six irradiated recipients, and the experiment was repeated with at least six WT and six Camk4−/− donors. The following day, bone marrow cells from three recipient mice per donor cell genotype were isolated and analyzed for the presence of CD45.2 marker to ensure “proper homing” of the donor KLS cells. To measure repopulation, peripheral blood was obtained from the retro-orbital vein every 3 weeks (17). The blood cells were labeled with CD45.1 FITC, CD45.2 PE, and either Mac-1 and Gr-1 for myeloid lineage or CD3 and B220 for lymphoid lineage analyses (16, 18).

Serial Bone Marrow Transplants—The primary recipient mice were sacrificed at 3.5 months. New CD45.1 recipient mice (n = 5/group) were irradiated (9.5 Gy in a single dose) and transplanted with 4 × 10^6 mononuclear bone marrow cells from sacrificed, individual primary recipient mice by injection via the retro orbital sinus. Bone marrow cells from each primary recipient were injected into five secondary recipients. Peripheral blood from secondary recipients was analyzed by flow cytometry every 3 weeks (17).

Bromodeoxyuridine (BrdUrd) Analysis—For in vivo BrdUrd labeling assays, WT and Camk4−/− mice were fed with 0.5 mg/ml of BrdUrd (Sigma) dissolved in drinking water for 4 days. KLS cells isolated from these mice were fixed in 70% ethanol at −20 °C, permeabilized, stained with BrdUrd-PE antibody according to manufacturer’s protocol (Pharmingen), and analyzed by fluorescence-activated cell sorting (FACS) for the presence of BrdUrd-PE-positive cells. For Ki-67 labeling, approximately 10,000 freshly sorted KLS cells were fixed in 80% ethanol for 12 h at −20 °C, permeabilized with 0.1% Triton X-100 (Sigma), and stained with FITC-labeled Ki-67 antibody (Pharmingen) for 30 min. The cells were then washed and subjected to FACS analysis for the presence of Ki-67–FITC-positive cells.

Annexin V Apoptosis Assay—Approximately 10,000 freshly isolated KLS cells were incubated with Annexin V–FITC and propidium iodine according to manufacturer’s instructions (Pharmingen). Stained cells were analyzed by flow cytometry within 30 min.

Immunocytochemistry—Freshly isolated KLS cells were collected onto slides by cytopsin, either immediately after isolation or after stimulation with 2 μM ionomycin or 3 μM forskolin (both from EMD Biosciences, La Jolla, CA) for 10 min at 37 °C. The cells were fixed in 4% paraformaldehyde for 30 min and permeabilized using 0.5% Nonidet P-40 for 10 min. Following overnight incubation at 4 °C with either anti-CREB NT (rabbit polyclonal, Upstate, Charlottesville, VA), anti-phospho-CREB (against Ser133, rabbit polyclonal, Upstate), anti-CBP (A-22, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl2 (mouse monoclonal, Pharmingen), or anti-p21waf1 (C-19G, goat polyclonal, Santa Cruz Biotechnology) the slides were incubated with the appropriate fluorescent secondary antibody. Digital confocal images were taken of all samples with the same settings and analyzed using Metamorph® software to quantify the intensity of the fluorescence; n > 50 for each condition.

Real-time RT-PCR Analysis—Total RNA was prepared from ~10,000 freshly isolated HSCs using the RNAqueous-Micro kit (Ambion, Austin, TX), according to manufacturer’s instructions. The first strand cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen), according to manufacturer’s directions. Quantitative real-time PCR-based gene expression analysis was performed using IQ SYBR Green Supermix with the respective primers, and the reactions were performed using a LightCycler (Roche Applied Science). The sequences of all the primers used in this study are available upon request.

Marine Stem Cell Virus (MSCV)-CamKIV Add-back Experiments—CamKIV-WT or CamKIV-K71M cDNA was cloned into MSCV-IRES-GFP vectors, and high titer control and recombinant viruses were made by pseudotyping with vesicular stomatitis virus glycoprotein. Approximately 30,000 WT or Camk4−/− KLS cells were allowed to proliferate overnight at 37 °C in X-vivo-15 (Cambrex, Walkersville, MD) media supplemented with 2% fetal bovine serum, 30 ng/ml stem cell factor, 30 ng/ml Flt-3 ligand, and 50 μM 2-mercaptoethanol. The cells were infected with the appropriate MSCV virus at an MOI of 5:1 and were harvested 3 days after infection. Expression of CamKIV-WT or CamKIV-K71M was confirmed by RT-PCR using specific primers against CamKIV. For in vitro cell proliferation assays, GFP−/MSCV-infected KLS cells were FACs sorted at 15 cells per well into Terasaki plates. The cells were grown in X-vivo-15 (Cambrex, Walkersville, MD) media supplemented with 5% FBS, 30 ng/ml stem cell factor, 30 ng/ml Flt-3 ligand, and 50 μM 2-mercaptoethanol for 6 days. The proliferation rate of the KLS cells was estimated by counting the number of cells in each well at the indicated time points. For immunocytochemistry, GFP− virus-infected KLS cells were cytopsin onto slides, fixed, and stained for respective antibodies as mentioned before. Protocols are available upon request.

RESULTS

Loss of CamKIV Results in Diminished Bone Marrow Cellularity and Number of HSCs—Initial histological analysis of bone sections from adult Camk4−/− mice revealed a decrease in bone marrow cellularity (Fig. 1, A and B), raising the possibility that CamKIV could play a role in hematopoiesis. To explore this idea, we analyzed peripheral blood samples from Camk4−/− mice and found a 44% decrease in total white blood cells (p < 0.005), a 43% decrease in cells of the myeloid lineage (neutrophils, monocytes, and eosinophils; p < 0.01), and a 53% decrease in lymphoid cells (T and B cells; p < 0.002) compared with WT (Fig. 1C). To evaluate whether hematopoietic progenitor activity is compromised in the absence of CamKIV, we performed colony forming...
assays on bone marrow cells isolated from WT and Camk4−/− mice. Bone marrow cells from Camk4−/− mice formed fewer granulocyte-monocyte and pre-B cell colonies compared with the WT (data not shown) suggesting that CaMKIV might regulate progenitor cell development.

Long term and short term HSCs and the multipotent progenitors primarily reside in the bone marrow where they differentiate into committed progenitors in the myeloid and lymphoid lineages, which further mature before release into the peripheral blood. Thus, a reduction in the number of peripheral blood cells could result either from a primary defect in HSC self-renewal/maintenance or in differentiation of these cells. To distinguish between these two possibilities, we first examined the frequency of KLS cells in WT and Camk4−/− mice by FACS. Fig. 1D shows that there is a 2-fold reduction in the number of KLS cells (12,000 per mouse on average) in Camk4−/− mice compared with WT (26,000 per mouse on average). These results raised the possibility that CaMKIV could participate in the maintenance of the KLS cell population in the bone marrow. To determine whether the absence of CaMKIV resulted in a higher number of KLS cells dying by apoptosis (19), freshly isolated KLS cells from Camk4−/− and WT mice were stained for AnnexinV and propidium iodide (PI). While the AnnexinV-positive:PI-negative population only includes intact cells that are in the early stages of apoptosis, the AnnexinV-positive:PI-positive population includes cells that are necrotic or at advanced stages of apoptosis as well as cells killed or damaged during isolation. FACS analysis revealed three times more AnnexinV-positive:PI-negative (early apoptotic) cells in the freshly isolated Camk4−/− KLS cell population compared with WT (Fig. 1E). Thus, the lower number of KLS cells in Camk4−/− mice and the position of these cells to apoptosis support the idea that CaMKIV has a role in maintaining KLS cell homeostasis by regulating their survival. Camk4−/− KLS Cells Are Compromised in Their Long Term Reconstitution Ability following Bone Marrow Transplantation — To investigate whether the role for CaMKIV in KLS cells is cell autonomous and whether CaMKIV deficiency compromises long term HSC function, we performed in vivo bone marrow reconstitution assays by injecting irradiated CD 45.1 recipient mice with ~1000 KLS cells isolated from CD 45.2 WT or Camk4−/− donor mice (20–22). We chose to transplant KLS cells rather than whole bone marrow as the latter could skew the results due to the lower frequency of KLS cells present in Camk4−/− mice. We first analyzed bone marrow cells from recipient mice 19 h after transplantation and confirmed that donor-derived KLS cells from WT and Camk4−/− mice equivalently “home” to the bone marrow of the host mice (Fig. 2Ai). Next, recipient reconstitution was determined by FACS analysis of peripheral blood samples drawn every 3 weeks (Fig. 2, Aii and B). Mice transplanted with WT cells displayed normal reconstitution patterns at 3, 6, 9, and 12 weeks after transplant (16). In contrast, KLS cells from Camk4−/− mice led to significantly enhanced peripheral blood reconstitution between 3 and 6 weeks after transplant (Fig. 2, Aii and B). However, by 9 weeks the percentage of donor-derived CD 45.2 Camk4−/− cells was markedly reduced in the peripheral blood of recipient mice relative to WT cells (Fig. 2, Aii and B).

Next, to determine whether the Camk4−/− donor KLS cells that remained in the bone marrow at 12 weeks post-transplantation were still functional, we performed secondary bone marrow transplantation assays. Approximately, 4 × 10^6 total bone marrow cells (containing 6000 CD45.2 WT-derived KLS cells or 200 CD45.2 Camk4−/−-derived
KLS cells) from recipient mice that had been transplanted 12 weeks previously with WT or Camk4−/− KLS cells were serially transplanted into new sub-lethally irradiated recipients. FACS analysis of blood samples drawn every 3 weeks showed no significant recipient reconstitution in mice transplanted with Camk4−/− cells, whereas reconstitution of WT cells occurred normally (Fig. 2C). Previous studies have shown that even as few as 1–10 viable long term HSCs are capable of reconstituting irradiated recipient bone marrow upon transplantation (18, 23). Cumulatively, these transplant data suggest that in contrast to the behavior of WT KLS cells, Camk4−/− KLS cells might inappropriately undergo a burst of engraftment followed by premature exhaustion, resulting in a loss of long term repopulating ability.

Absence of CaMKIV Results in Enhanced in Vivo and in Vitro Proliferation by KLS Cells and Re-expression of CaMKIV, but Not a Kinase-defective Mutant, Rescues This Defect in Vitro—The enhanced engraftment of Camk4−/− KLS cells early after the transplant could be attributed to an enhanced proliferation by the Camk4−/− KLS cells in the bone marrow of the irradiated recipient mice. To test this possibility.
Role of CaMKIV in Hematopoietic Stem Cell Self-renewal

we performed in vivo labeling of WT and Camk4−/− mice with BrdUrd for 4 days. The KLS cells were then isolated, fixed, stained with anti-BrdUrd antibody, and analyzed by FACS. While only 9% of the WT KLS cells were positive for BrdUrd incorporation, about 30% of Camk4−/− KLS cells stained positive for BrdUrd (Fig. 3A), indicating that a higher number of mutant cells are in proliferation. We also confirmed that Camk4−/− KLS cells have a greater proliferation index by Ki-67 labeling of freshly isolated KLS cells (data not shown). These results indicate that CaMKIV might act to suppress excessive proliferation by KLS cells in the bone marrow, thereby regulating HSC homeostasis.

If the role of CaMKIV in KLS cells is to suppress inappropriate cell proliferation, then re-expression of CaMKIV in Camk4−/− KLS cells should rescue their hyperproliferative phenotype. To test this idea we introduced either wild type CaMKIV (CaMKIV-WT) or a kinase-inactive CaMKIV (CaMKIV-K71M) into WT and Camk4−/− KLS cells using the MSCV-based vector that also encodes the GFP under the control of an IRES downstream of the cloned gene. KLS cells infected with the viruses were sorted based on GFP expression, and the expression of CaMKIV in WT KLS cells and its introduction into Camk4−/− KLS cells through MSCV infection was confirmed by RT-PCR (Fig. 3B). Equal numbers of GFP-positive WT and Camk4−/− KLS cells were then plated on Terasaki plates in media supplemented with 5% fetal bovine serum, 30 ng/ml stem cell factor and 30 ng/ml Flt-3 ligand. Proliferation was followed by counting the number of cells at 2-day intervals. On days 2 and 4 of in vitro growth, Camk4−/− KLS cells infected with MSCV-control virus proliferate at a higher rate (2-fold higher) than WT KLS cells (Fig. 3C). In addition, Camk4−/− cells are exhausted to a greater extent than the WT cells between days 4 and 6. Remarkably, re-expression of CaMKIV rescues both the hyperproliferation as well as the rapid exhaustion phenotypes characteristic of Camk4−/− KLS cells, and these cells behave in a similar fashion to WT cells infected with the control virus (Fig. 3C). CaMKIV activity is required for the rescue of the proliferation defects in the Camk4−/− KLS cells as the kinase-inactive mutant (K71M) was not able to alter proliferation of the mutant cells (Fig. 3C). In fact, not only did CaMKIV-K71M fail to reduce proliferation to WT levels but also hyperproliferation was exacerbated in the mutant cells, such that at 4 days of in vitro growth, there were more Camk4−/− cells expressing CaMKIV-K71M than Camk4−/− cells infected with the control virus. These data indicate that CaMKIV can regulate HSC proliferation in vitro and suggest that the enhanced engraftment observed in vivo is due to the overproliferation of Camk4−/− KLS cells. Cumulatively these findings indicate that CaMKIV activity is important for maintaining HSCs in a relatively quiescent state.

Lower Levels of Phospho-CREB, CBP, and Bcl-2 mRNA and Protein Levels in Camk4−/− KLS Cells—What is the signaling pathway by which CaMKIV functions to maintain hematopoietic homeostasis? CaMKIV can phosphorylate CREB on Ser133 (pCREB) in response to transient increases in intracellular calcium (24). Since decreased levels of pCREB and CBP have been found in neurons (13, 14) and memory T cells (1) of Camk4−/− mice, we examined whether decreased Ca2+-induced pCREB was also observed in Camk4−/− KLS cells. As shown in Fig. 4, Ai and Aii, pCREB was reduced 2.5-fold in KLS cells deficient in CaMKIV as determined by immunofluorescence. In addition, whereas pCREB in WT cells was increased substantially following ionomycin treatment, very little increase was noted in similarly treated Camk4−/− cells (supplemental Fig. 1). As total CREB levels were similar in WT and Camk4−/− mice (Fig. 4, Ai and Aii) these results indicate that the Ca2+ signaling pathway leading to pCREB must be active in KLS cells in vivo and suggest that a defect in CaMKIV/pCREB-mediated transcription may compromise the functions of these cells.

Phosphorylation of CREB on Ser133 is required to recruit the CREB-binding proteins CBP or p300 to transcription complexes, which is in turn required for transcriptional activation of CRE-containing promoters (1, 9, 25, 26). In addition to phosphorylating CREB, CaMKIV has also been reported to phosphorylate CBP on Ser301 (20), which positively regulates its function as a transcriptional co-activator. Although antibodies specific to CBP-pSer301 that can be used in immunocytochemistry are unavailable, we did use a CBP polyclonal antibody to evaluate whether or not CBP levels might be altered in Camk4−/− KLS cells. Surprisingly, CBP is significantly reduced, by 2.4-fold, in the Camk4−/− KLS cells compared with WT cells (Fig. 4, Ai and Aii). These data raise the possibility that phosphorylation of CREB and/or CBP by CaMKIV might play a role in maintaining CBP levels in these cells and support the idea that a Ca2+-dependent CaMKIV/CREB/CBP signaling cascade is active in HSCs.

If a CaMKIV signaling cascade functions through CREB and CBP to regulate transcription in HSCs, what target gene or genes might be activated to suppress proliferation as well as enhance survival of KLS stem cells? To explore possible mechanisms by which CaMKIV might regulate KLS proliferation and homeostasis, we compared the mRNA levels of several pro- and anti-apoptotic genes as well as the cyclin-dependent kinase inhibitor, p21cip1 in WT and Camk4−/− KLS cells. As shown in Fig. 4, B and C, the absence of p21cip1 has previously been shown to result in hematopoietic stem cell exhaustion upon serial bone marrow transplantation (17). Our results reveal that, among the 14 mRNAs evaluated, only the Bcl-2 mRNA is differentially expressed between WT and Camk4−/− KLS cells, and as shown in Fig. 4, B and C, this 1.9-fold decrease in the Camk4−/− KLS cells is statistically significant. Several studies have shown that transcription of the pro-survival gene Bcl-2 gene requires pCREB and can be stimulated by Ca2+ (27–29). Moreover, in addition to its role in cell survival, Bcl-2 has been reported to play a role in maintaining cellular quiescence (30, 31). We also examined Bcl-2 protein levels in freshly isolated WT and Camk4−/− KLS cells by immunocytochemistry. Bcl-2 protein levels are 2.7-fold lower in
Camk4\(^{-/-}\) KLS cells compared with the WT cells (Fig. 4, Ai and Aii), while protein levels of the cyclin-dependent kinase inhibitor p21\(^{CIP1}\) are similar in WT and Camk4\(^{-/-}\) KLS cells (Fig. 4, Ai and Aii). Collectively, our data indicate that in freshly isolated KLS cells, there is a positive correlation between the presence of CaMKIV, phosphorylation of CREB, and levels of CBP, Bcl-2 mRNA, and Bcl-2 protein.

Since freshly isolated Camk4\(^{-/-}\) KLS cells show a 2.5-fold reduction in pCREB levels compared with WT cells, we wondered whether culturing these cells in the presence of stimuli that activate Ca\(^{2+}\)-independent CREB kinases would increase pCREB in Camk4\(^{-/-}\) cells. Consistent with this idea, we could increase pCREB to the same level in freshly isolated Camk4\(^{-/-}\) and WT KLS cells following incubation with forskolin (supplemental Fig. 1), demonstrating that the cAMP-dependent protein kinase pathway is intact and leads to CREB phosphorylation in both cell types.

Re-expression of CaMKIV Results in Restoration of WT Levels of CBP and Bcl-2 in Camk4\(^{-/-}\) KLS Cells—We hypothesized that if the loss of CaMKIV in KLS cells is specifically responsible for decreased pCREB, CBP, and Bcl-2, then re-expression of Camk4 in freshly isolated Camk4\(^{-/-}\) KLS cells might reverse these defects by reconstituting the Ca\(^{2+}\)-dependent signaling pathway. As shown in Fig. 5, A and B, respectively, Bcl-2 and CBP levels were 3.5- and 2-fold lower in Camk4\(^{-/-}\) KLS cells infected with MCSV-control virus, compared with control virus-infected WT cells. Re-expression of CaMKIV-WT, but not kinase inactive CaMKIV-K71M, quantitatively restores WT levels of CBP and Bcl-2 in the Camk4\(^{-/-}\) KLS cells (Fig. 5, A and B). Interestingly, pCREB levels were only slightly reduced in Camk4\(^{-/-}\) KLS cells infected with the control virus compared with WT cells and introduction of either CaMKIV-WT or CaMKIV-K71M resulted in only a slight but non-significant increase in pCREB levels in these cells (Fig. 5, A and B). We suspect that normalization of CREB phosphorylation in both cell types is due to serum-induced activation of CREB kinases other than CaMKIV as illustrated by the Forskolin experiments above (supplemental Fig. 1). These results also show that pCREB may be necessary but is not sufficient to restore Bcl-2 gene expression in the absence of CaMKIV and support the idea that an important component of the action of CaMKIV is an effect on CBP (9, 10). At any rate when taken together, our data support a role for a Ca\(^{2+}\)/CaMKIV/pCREB/CBP pathway in the regulation of Bcl-2 gene expression in KLS cells and strengthen our idea that this pathway may be important for promoting maintenance of HSC pool in mouse bone marrow by preventing inappropriate proliferation of KLS cells.

DISCUSSION

Self-renewing hematopoietic stem cells replenish billions of mature myeloid and lymphoid cells in the blood on a daily basis, a process that is vital for sustaining life. Understanding how the molecular regulation of HSC self-renewal is achieved is crucial for the improvement of HSC-based transplantation therapies. We have uncovered a novel role for CaMKIV in the maintenance of HSC homeostasis. The CaMKIV gene is expressed in KLS cells (Fig. 3B, lanes 1 and 2), and its absence results in lower numbers of these cells in the bone marrow of null animals. We
also find that the absence of CaMKIV results in increased proliferation of KLS cells. When Camk4−/− mice KLS cells are challenged with expansion signals in vivo (bone marrow transplantation) or in vitro (growth factor-containing medium) they undergo premature proliferation followed by exhaustion. At least in culture, these altered proliferation defects could be rescued by re-expression of CaMKIV in the Camk4−/− KLS cells implying that, even acutely, this protein kinase serves as an inhibitor of inappropriate proliferation of the HSCs. The number of self-renewing HSCs in the bone marrow is regulated, at least in part, due to elimination of excess HSCs generated through inappropriate proliferation by apoptosis (32). Since the absence of CaMKIV results in increased proliferation, as well as in predisposition of the cycling cells to exhaustion via apoptosis, we suggest that CaMKIV plays a role in HSC homeostasis.

The Camk4−/− mice utilized in this study are asymptomatic and live a normal life span when housed in a clean environment. The difference in the number of KLS cells between WT and Camk4−/− mice is 2-fold under homeostatic conditions, which may not be sufficient to cause overt immunological abnormalities. However, when challenged with stress or expansion signals such as those presented by bone marrow transplantation, these differences became much more important (Fig. 2, A–C). Similar results due to relatively small differences in KLS cell number under homeostasis that become amplified upon being challenged have been observed in Bcl-2 transgenic, p21cip1−/− and p18ink4c−/− mice (17, 32, 33). Additionally, the zinc finger transcriptional repressor Gfi-1 was recently shown to be a regulator of HSC proliferation (34, 35). Although HSCs from both Camk4−/− and Gfi-1−/− mice show higher proliferation, unlike the Camk4−/− mice, Gfi-1−/− mice have a higher number of KLS cells in their bone marrow under homeostatic conditions (34, 35). However, similar to Camk4−/− KLS cells, Gfi-1−/− HSCs fail to reconstitute the bone marrow upon serial transplantation, due to their exhaustion in response to the expansion stimulus provided by transplantation (34).

Freshly isolated Camk4−/− KLS cells have significantly lower levels of pCREB, CBP, Bcl-2 mRNA, and Bcl-2 protein levels compared with WT cells. CBP and Bcl-2 levels could be restored by re-expression of CaMKIV, whereas culturing cells in the presence of serum and growth factors increased pCREB levels in KLS cells whether or not CaMKIV was present. These results suggest that pCREB may be necessary but is not sufficient for maintaining CBP and Bcl-2 in the absence of CaMKIV. As such, CBP and Bcl-2 levels may be restored by re-expression of CaMKIV, whereas cultures cells in the presence of serum and growth factors increased pCREB levels in KLS cells whether or not CaMKIV was present.
gence strongly suggests a role for a Ca\(^{2+}\)/CaMKIV/pCREB/CBP pathway in the regulation of Bcl-2 expression in KLS cells.

Consistent with the idea of a role for a CaMKIV, CBP, Bcl-2 pathway in the regulation of the HSC population, targeted overexpression of Bcl-2 in HSCs in vivo results in increased number, quiescence, and self-renewal of HSCs (32), precisely the opposite phenotypes that we report herein to arise due to the absence of CaMKIV. Thus, in the Bcl-2 transgenic mouse, although a higher percentage of HSCs is quiescent, the steady-state number of HSCs is actually increased as a result of a failure of these cells to be cleared by apoptosis (due to enhanced Bcl-2 expression) (32).

Mice haplo-insufficient for CBP also exhibit HSC exhaustion (36, 37). Although silencing of the CBP gene results in early embryonic lethality, mice heterozygous for CBP null mutation survive and display multiple severe phenotypes (37). Interestingly, CBP haplo-insufficient mice and CaMKIV null mice share phenotypic consequences in the brain and hematopoietic systems, although these two types of genetically altered mice do not phenocopy each other (36–38). One example of a difference between the two mouse lines is that the CBP\(^{-1/2}\) mice show age-dependent decrease in bone marrow cellularity and decrease in numbers of KLS hematopoietic stem cells as well as numbers of myeloid and B cell colony forming progenitors in the bone marrow (36). Second, HSCs from CBP\(^{-/-}\) mice show no reconstitution only after tertiary bone marrow transplantation (37). Finally, mice heterozygous for CBP null mutation survive and display multiple phenotypic consequences in the brain and hematopoietic system. Although silencing of the CBP gene results in early embryonic lethality, mice heterozygous for CBP null mutation survive and display multiple phenotypic consequences in the brain and hematopoietic system (19, 20).

Based on this collective evidence, we suggest that a Ca\(^{2+}\)/CaMKIV/CREB/CBP signaling pathway is critical for the maintenance of HSC homeostasis and that one target for this pathway is likely to be the Bcl-2 gene (Fig. 5C). Unquestionably, additional genes regulated by this pathway collectively contribute to the regulation of HSC self-renewal by CaMKIV and we are actively pursuing their identity. Nevertheless, our data argue that decreased levels of Bcl-2, a protein with dual roles in the maintenance of cell survival and cell quiescence (31), may be an important contributing factor for the inability of the HSCs of the Camk4\(^{-/-}\) mice to maintain quiescence and for the inappropriate proliferation of these cells when challenged with an expansion signal. In addition, the decrease in Bcl-2 in these proliferating HSCs might result in increased susceptibility of this cell population to apoptosis and together these events result in the eventual exhaustion of the hematopoietic stem cells.

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