CaM Kinase IV Regulates Lineage Commitment and Survival of Erythroid Progenitors in a Non-Cell–Autonomous Manner

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Abstract. Developmental functions of calmodulin-dependent protein kinase IV (CaM KIV) have not been previously investigated. Here, we show that CaM KIV transcripts are widely distributed during embryogenesis and that strict regulation of CaM KIV activity is essential for normal primitive erythropoiesis. Xenopus embryos in which CaM KIV activity is either upregulated or inhibited show that hematopoietic precursors are properly specified, but few mature erythrocytes are generated. Distinct cellular defects underlie this loss of erythrocytes: inhibition of CaM KIV activity causes commitment of hematopoietic precursors to myeloid differentiation at the expense of erythroid differentiation, on the other hand, constitutive activation of CaM KIV induces erythroid precursors to undergo apoptotic cell death. These blood defects are observed even when CaM KIV activity is misregulated only in cells that do not contribute to the erythroid lineage. Thus, proper regulation of CaM KIV activity in nonhematopoietic tissues is essential for the generation of extrinsic signals that enable hematopoietic stem cell commitment to erythroid differentiation and that support the survival of erythroid precursors.

Key words: Xenopus • CaM KIV • hematopoiesis • erythropoiesis • embryogenesis

Introduction

During embryogenesis, a myriad of extracellular signals work together to pattern the embryo. In many cell types, extracellular stimuli regulate cell processes by elevating the level of intracellular calcium, thereby activating the ubiquitous calcium-binding protein: calmodulin (CaM).1 The Ca2+-CaM complex binds to and modulates the properties of numerous proteins, including a family of CaM-dependent serine/threonine protein kinases (for review see Soderling, 1996). These kinases share certain structural motifs, such as a conserved catalytic domain positioned adjacent to overlapping autoinhibitory and CaM-binding domains. At low concentrations of intracellular calcium, the autoinhibitory sequence interacts with the catalytic core, to be phosphorylated by a CaM-dependent kinase kinase (CaM KK). Phosphorylation of this Thr 196 by CaM KK results in a 10–20-fold activation of CaM KIV. In addition to CaM KIV, CaM KK has two other known substrates: CaM-dependent kinase I (CaM KI) (Haribabu et al., 1995; Soderling, 1996) and protein kinase B (Yano et al., 1998).

The substrate specificity of CaM KIV is not well defined, but it has been shown to phosphorylate and regulate various transcription factors (for review see Soderling, 1999). Consistent with a role in regulating transcription, CaM KIV and CaM KK show strong nuclear localization, but are also present in the cytosol (Sakagami et al., 1999). By contrast, CaM KI appears to be only cytosolic (Picciotto et al., 1995). In addition to responding to intracellular calcium, the CaM kinase cascade participates in cross talk with other signaling cascades. For example, CaM KIV directly phosphorylates and inhibits the activity of type I adenyl cyclase...
In adult mammals, CaM KIV is expressed predominantly in a subset of neurons, in T lymphocytes and in male germ cells (for review see Means et al., 1997). In the immune system, CaM KIV appears to be required to prevent apoptosis during T cell development and may function in the cascade of events that is required for activation of mature T cells (Anderson et al., 1997; for review see Means et al., 1997). CaM KIV also plays a role in learning and memory within the brain (Bito et al., 1996; Ahn et al., 1999).

Little is known about embryonic expression patterns or developmental roles of CaM KIV. To elucidate such function(s), we examined the consequences of inactivating or constitutively activating this signaling pathway in *Xenopus* embryos. Misregulation of CaM KIV activity led to several developmental defects. In this study, we have focused on the role of CaM KIV in primitive hematopoiesis.

Vertebrate hematopoiesis is a multistep process in which pluripotent, self-renewing stem cells commit to and ultimately differentiate along one of the various mature lineages of the blood (for review see Zon, 1995; Evans, 1997). In *Xenopus*, blood cell progenitors can arise from any blastomere of the 16-cell embryo (Mills et al., 1999). These cells will populate the ventral blood island (VBI, which is the functional equivalent of the yolk sac blood island of higher vertebrates), which gives rise to all progenitors of primitive hematopoiesis (Turpen et al., 1997). Primitive hematopoiesis is carried out by a transient population of stem cells and differs significantly from adult, definitive hematopoiesis. Whereas primitive hematopoiesis produces predominantly embryonic erythrocytes (red blood cells [RBCs]), definitive hematopoiesis yields adult erythroid cells, as well as progenitors of the lymphoid (lymphocyte) and myeloid (granulocyte, monocyte, and platelet) lineages. In *Xenopus* embryos, definitive hematopoietic cells are derived from the VBI and from the mesoderm of the dorsal–lateral plate (Kau and Turpen, 1983; Máeno et al., 1985; Weber et al., 1991; Turpen et al., 1997).

The induction, proliferation, and differentiation of blood progenitors is directed by cues from nonhematopoietic tissues and by signals intrinsic to the hematopoietic stem cells (HSCs) themselves. For example, bone morphogenetic protein-4 specifies ventral fate within the mesoderm, enabling blood progenitors to be born (for review see Lemaire and Yasuo, 1998), and may subsequently regulate the proliferation and differentiation of primitive hematopoietic cells (Bhatia et al., 1999). Unidentified signals provided by endodermal (Yoder et al., 1994; Belaoussoff et al., 1998) and endothelial cells (Yoder et al., 1994; Fennie et al., 1995; Ohneda et al., 1998) can also influence the proliferation, survival, and/or lineage fate of HSCs. Downstream of these extrinsic signals, a regulatory network of hematopoietic-specific transcription factors functions in the specification and further development of blood progenitors (Huber and Zon, 1998; for review see Sieweke and Graf, 1998).

In this study, we found that the proper regulation of CaM KIV activity in nonhematopoietic tissues is essential for hematopoietic progenitors to commit to the erythroid lineage and for the survival of erythroid precursors.

### Materials and Methods

#### Isolation of *Xenopus* CaM Kinase IV cDNAs

Total RNA was prepared from stage 45 embryos and reverse transcribed, as described previously (Cui et al., 1996). Degenerate PCR primers were designed based on sequence motifs that are conserved between human, mouse, and rat CaM KIV. A partial-length cDNA encoding a portion of the catalytic domain of CaM KIV was amplified using the following nested paired oligonucleotides: outside forward primer: 5'-TTT GAA TTC AAR GAR AAR GGN TAY TA-3' (R, purine; Y, pyrimidine; N, any nucleotide) (coding for KEIFET, amino acids 134–139 of the mouse CaM KIV), inside forward primer: 5'-TTT CAA TTC GTN GAR AAR GGN TAY TA-3' (coding for VEKGY, amino acids 162–167 of mouse CaM KIV), inside reverse primer: 5'-GGG TCT AGA RAA CAT RAA YTG RTC RTC-3' (coding for GDQMF, amino acids 277–282 of mouse CaM KIV), outside reverse primer: 5'-GGG TCT AGA NAC YTC RTC CCA CCA NGG-3' (coding for PWWDVE, amino acids 295–300 of mouse CaM KIV). PCR products were subcloned into pGEMT®-EZ T and sequenced on both strands. From these sequences, appropriate gene specific primers were constructed and full-length CaM KIV cDNAs were isolated using 5’ and 3’ RACE. High fidelity polymerase was used in all PCRs and multiple independent clones were fully sequenced on both strands to determine the correct coding sequence.

#### Generation of Mutant Forms of *Xenopus* CaM KIV

The constitutively active *Xenopus* CaM KIV cDNA (CaM KIVc) was generated by introducing point mutations such that the sequence encoding HMDN (amino acids 313–316) (see Fig. 1) within the autoinhibitory domain was changed to DMDD. Introduction of these acidic charged mutations inactivates the autoinhibitory domain, generating a calcium-independent kinase (Tokumitsu et al., 1994). The dominant-negative CaM KIV cDNA (DnCaM KIV) encodes a protein containing the acidic charged mutations present in CaM KIVc, in addition to the following mutations: (a) Lys 79 in the ATP-binding site was mutated to Gln and (b) the activation loop phosphorylation site (Thr 204) was mutated to Ala. Mutations were introduced by PCR-mediated amplification of the *Xenopus* CaM KIV cDNA using primers that contained appropriate nucleotide substitutions. PCR-generated portions of mutant cDNAs were sequenced and all cDNAs were subcloned into the expression vector pCS2+ (Rupp et al., 1994).

#### Embryo Culture and Manipulation

*Xenopus* eggs were obtained, injected with plasmid DNA or synthetic mRNAs, and cultured as described previously (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (1967). Capped synthetic RNA was generated by in vitro transcription of linearized template cDNAs using a Megascript Kit (Ambion). In initial experiments, embryos were injected with RNAs encoding mouse DnCaM KIV or mouse CaM KIVc together with constitutively active rat CaM KK (Enslen et al., 1996). After in situ hybridization, some embryos were dehydrated in methanol, embedded in paraffin, and sections 14–20-μm thick were cut and counterstained with eosin (Christian and Moon, 1993).

#### β-Galactosidase Staining, In Situ Hybridization, and Histological Analysis

Embryos were stained for β-galactosidase activity using Red-gal (Research Organics, Inc.) as a substrate and processed for in situ hybridization, as described previously (Nakayama et al., 1998). After in situ hybridization, some embryos were dehydrated in methanol, embedded in paraffin, and sections 14–20-μm thick were cut and counterstained with eosin (Christian and Moon, 1993).

#### Northern Blot and Reverse Transcription–PCR Analysis of Gene Expression

RNA was isolated and reverse transcription (RT)-PCR analysis was performed, as described previously (Cui et al., 1996), using the following PCR conditions: one cycle at 95°C for 5 min, followed by cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The cycle number was determined empirically for each primer pair so that PCR products were examined during the exponential phase of amplification. The following primer pairs (in 5’ to 3’ orientation) were used to amplify CaM KIV: upstream GGA ATG CTG...
CAG ATG CTG and downstream CAT GGA CTG TCC TCG. For globin, the primers were: upstream TTG CTG TCT CAC ACC ATC CAG G and downstream TCT GTA CTT GGA GGT GAG GAC G. Ornithine decarboxylase (ODC) (Nastos et al., 1998) and GATA-1 (Zon et al., 1991) primers have been published. Northern blot hybridization was performed as described previously (Christian and Moon, 1993). Radioactive bands were visualized with a Molecular Dynamics phosphorimager, and the Macintosh IP lab gel program was used to quantitate relative levels of expression of each gene.

Western Blot Analysis and Activity Assays of CaM KK

Proteins were extracted from Xenopus embryos, as described previously (Moon and Christian, 1989), and analyzed by SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti–rat CaM KK or CaM KIV antibodies (Transduction Labs). Immunocomplexes were visualized using a mouse HRP-conjugated secondary antibody and a chemiluminescent detection system (NEN Life Science Products). CaM KK activity in embryo extracts was measured and normalized relative to the total protein content, as described in Wayman et al. (1997).

Collection and Analysis of Peripheral Blood Samples

Tails were severed from tadpole stage embryos and hematopoietic cells were collected into amphibian PBS containing 0.5% BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytocentrifuge. For general morphological examination, blood cells were stained with a Hema 3 stain set (Biochemical Sciences Inc.) and examined by light microscopy. Apoptotic cells in cytospin preparations of peripheral blood were detected by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay using a fluorescein apoptosis detection kit (Promega). Nuclei of cells were counterstained with propidium iodide to determine the total number of cells.

Results

Molecular Cloning and Developmental Expression of Xenopus CaM KIV

A cDNA encoding Xenopus CaM KIV (xCaM KIV) was obtained using a PCR-based strategy. A comparison of the predicted sequence of xCaM KIV protein with that of its murine ortholog (mCaM KIV) is shown in Fig. 1. Mouse and Xenopus CaM KIV share a high degree of homology within their catalytic- (96% amino acid identity, shaded box) and autoinhibitory/CaM-binding domains (93% amino acid identity, open box). xCaM KIV contains a non-conserved serine/threonine repeat insert at its NH$_2$ terminus and the COOH terminus is truncated relative to mCaM KIV.

Transcripts encoding CaM KIV are present in Xenopus eggs and persist at a uniform level throughout embryonic development (Fig. 2 A). Analysis of RNA isolated from various dissected regions of embryos revealed that CaM KIV transcripts are distributed fairly uniformly across the dorsal–ven-
The spatial pattern of expression of CaM KIV was further examined by whole-mount in situ hybridization of digoxigenin-labeled riboprobes to embryos at different stages of development (Fig. 3). This analysis confirmed that CaM KIV transcripts are broadly distributed in early gastrula (Fig. 3 A) through late neurula- (B) stage embryos. In tailbud stage 34 (Fig. 3 C) and tadpole stage 39 (D–G) embryos, CaM KIV transcripts are highly enriched in lateral regions of the midbrain (MB) and hindbrain (HB), the olfactory placode (OP), Rohon-Beard sensory neurons (R-B), and in various cranial nerves, including the ophthalmic branch of the trigeminal nerve (CN V), the facial nerve (CN VII), and the glossopharyngeal nerve (CN IX). Although RT-PCR analysis demonstrates that CaM KIV transcripts are present in ventral cells of tailbud-stage embryos (Fig. 2 B), this is not readily apparent by in situ hybridization, most likely because of a low level ubiquitous expression and the fact that ventral cells have a high yolk content that prevents efficient penetration of probes (Lemaire and Gurdon, 1994).

**Enzymatically Active CaM KK Is Present throughout Embryogenesis**

CaM KK, the upstream activator of CaM KIV, is also expressed embryonically. As shown in Fig. 4, the temporal and spatial pattern of expression of embryonic CaM KK mirrors its activity profile. Antisera directed against rat CaM KK detected an immunoreactive band of \( \sim 68 \) kD that comigrated with recombinant rat CaM KK\( \alpha \) on Western blots of *Xenopus* egg and embryo extracts. This protein increased in abundance by tadpole stage 42, at which time a second immunoreactive protein of \( \sim 70 \) kD was also
detected. The size of this higher molecular weight species is consistent with it being the *Xenopus* ortholog of CaM KKβ. The increase in CaM KK protein levels at the tadpole stage correlated with an approximately threefold increase in CaM KK activity. To determine whether CaM KK activity is spatially restricted, we analyzed protein expression and activity in extracts isolated from dissected dorsal or ventral halves of early (stage 10) gastrulae. CaM KK protein and activity were detected in dorsal and ventral halves of embryos.

**Proper Regulation of CaM KIV Is Required for Normal Erythropoiesis**

To analyze embryonic functions of CaM KIV, we used previously characterized constitutively active (CaM KIVc) (Enslen et al., 1996) or dominant-negative (DnCaM KIV) forms of CaM KIV to misregulate its activity in developing *Xenopus* embryos. DnCaM KIV blocks the activation of cotransfected CaM KIV by CaM KK in transient transfection assays (Gringhuis et al., 1997) and has been used to block the function of endogenous CaM KIV in vivo (Gringhuis et al., 1997; Impey et al., 1998; Ahn et al., 1999). Inhibition of embryonic CaM KIV was achieved by microinjecting RNA (1 ng) encoding DnCaM KIV into specific blastomeres of cleaving embryos, whereas activation was achieved by coinjecting RNAs (100–200 pg each) encoding CaM KIVc and constitutively active CaM KIV (CaM KKc). Coexpression of these two constitutively active kinases generates eight to tenfold greater calcium-independent CaM KIV activity than does expression of CaM KIVc alone (Enslen et al., 1996). In all experiments, identical results were obtained by injection of ~10-fold more RNA (1–2 ng) encoding CaM KIVc alone (data not shown), indicating that the effects were due to activated CaM KIV, rather than another target of CaM KK. Injection of RNA encoding DnCaM KIV or CaM KIVc led to the accumulation of ectopic protein at levels greater than endogenous levels, at least until tailbud stage 28 (Fig. 5 A).

When CaM KIV activity was upregulated or inhibited in ventral cells, by injection of appropriate RNAs near the ventral marginal zone (VMZ) at the four-cell stage, embryos appeared morphologically normal at tadpole stage 46, except that their hearts appeared pale and very few circulating RBCs were present (data not shown). This same apparent anemia was noted when RNA encoding constitutively active or DnCaM KIV was injected near the dorsal marginal zone (DMZ), but in this case distinct abnormalities of the gut were also observed (Fig. 5 B, top row).

**Table I. Misregulation of CaM KIV in a Subset of Embryonic Cells Inhibits Expression of Globin throughout the VBI**

| RNA       | Injection site | Expression of globin in the VBI |
|-----------|----------------|---------------------------------|
|           |                | Decreased or absent | Strong |
|           |                | Throughout | Posterior | Anterior | Throughout | n   |
| DnCaM KIV | DMZ bilat.     | 66         | 17        | 17       | 0          | 48  |
| DnCaM KIV | DMZ unilat.    | 75         | 2         | 8        | 15         | 48  |
| DnCaM KIV | VMZ bilat.     | 89         | 0         | 0        | 11         | 65  |
| DnCaM KIV | VMZ unilat.    | 80         | 10        | 0        | 10         | 40  |
| CaM KKc/KIVc | DMZ bilat.     | 59         | 4         | 22       | 15         | 27  |
| CaM KKc/KIVc | DMZ unilat.     | 64         | 7         | 22       | 7          | 56  |
| CaM KKc/KIVc | VMZ bilat.     | 66         | 11        | 7        | 16         | 73  |
| CaM KKc/KIVc | VMZ unilat.     | 50         | 31        | 19       | 0          | 32  |
| DnCaM KI  | VMZ bilat.     | 21         | 4         | 4        | 71         | 52  |
| None      | None           | 5          | 9         | 7        | 79         | 114 |

RNAs were injected into one (unilat., unilateral) or two (bilat., bilateral) blastomeres of four-cell embryos near the DMZ or the VMZ. Expression of *globin* was analyzed by in situ hybridization at stage 32. Embryos were scored for decreased or absent staining predominantly in the anterior or posterior portions of the VBI or throughout the entire VBI. Numbers are expressed as percents, except for *n*, which denotes sample size.
which will be described in detail elsewhere (Wayman, G.A., and J.L. Christian, manuscript in preparation). In Xenopus, RBCs begin to differentiate in the VBI at the tailbud stage and can be identified by the expression of globin. Embryos in which CaM KIV activity was either up-regulated or inhibited showed a dramatic reduction in globin expression, as analyzed by whole-mount in situ hybridization (Fig. 5 B, bottom two rows). Anterior and posterior portions of the VBI are derived from dorsal and ventral regions of the embryo, respectively (Tracey et al., 1998), although lineage analysis has also shown that individual blastomeres of cleavage-stage embryos can contribute to the entire anteroposterior extent of the blood island (Mills et al., 1999). We did not observe a strong correlation between the site of injection of mutant CaM KIV RNA (DMZ versus VMZ) and the region of the VBI (anterior versus posterior) in which globin expression was absent. Rather, staining was completely lost or greatly diminished throughout the entire extent of the VBI in most experimental embryos (Fig. 5 B; Table I). Furthermore, when mutant CaM KIV RNA was injected unilaterally into a single blastomere near the DMZ or VMZ, such that ectopic RNA was segregated primarily to the right or left half of the embryo, globin staining was again decreased or absent throughout right and left halves of the VBI in most embryos (Table I).

As a control for specificity of inhibiting the CaM KIV arm of the CaM KK cascade, we injected RNA (1 ng) encoding dominant-negative CaM KI (DnCaM KI) near the VMZ of four-cell embryos. Over 70% of these embryos showed strong expression of globin throughout the VBI at the tailbud stage, comparable to un.injected controls,
whereas only 11% of embryos in which CaM KIV was inhibited showed strong expression of globin (Table I).

Northern blot analysis confirmed that constitutive activation or inhibition of CaM KIV activity on either the dorsal or ventral side of embryos led to a dramatic decrease in globin transcripts (Fig. 5 C). Tailbud stage 29 embryos in which CaM KIV activity was misregulated expressed globin at only 16–32% of control levels, and expression remained substantially repressed in stage 39 tadpoles.

CaM KIV Plays a Non-Cell–Autonomous Role in Regulating Hematopoiesis

Both dorsal and ventral blastomeres of cleavage-stage Xenopus embryos contribute to the VBI (Tracey et al., 1998; Mills et al., 1999; Lane and Smith, 1999), which might explain why misregulation of CaM KIV activity in dorsal cells disrupts globin expression in a ventral region. However, our finding that expression of globin is almost completely repressed when CaM KIV activity is perturbed in only a subset of embryonic cells (Fig. 5, B and C; Table I) raised the possibility that CaM KIV functions non-cell–autonomously in blood development. Specifically, CaM KIV may be required in nonhematopoietic cells to regulate the generation of extrinsic signals that direct normal erythropoiesis. To test this hypothesis, we targeted RNAs encoding mutant CaM KIV to blastomeres that do not contribute to the RBC lineage and then analyzed expression of globin at the tailbud stage. As illustrated in the diagram

Table II. Misregulation of CaM KIV in Nonhematopoietic Cells Inhibits Globin Expression

| RNA               | Injection site | 0 | + | ++ | +++ | n |
|-------------------|----------------|---|---|----|-----|---|
| CaM KKc/KIVc      | A1             | 16| 34| 30 | 20  | 88 |
| CaM KKc/KIVc      | A4             | 5 | 32| 42 | 21  | 38 |
| β-galactosidase   | A1             | 0 | 8 | 31 | 61  | 78 |
| None              |                | 3 | 0 | 28 | 69  | 58 |
| DnCaM KIV         | A1             | 24| 49| 12 | 15  | 67 |
| DnCaM KIV         | A4             | 10| 50| 30 | 10  | 40 |

RNA encoding β-galactosidase was injected alone or together with RNA encoding mutant CaM kinase into A1 or A4 blastomeres as illustrated above in Fig. 6 A. Expression of globin was analyzed by in situ hybridization at stage 32 and was scored as follows: +++, strong staining; ++, slight decrease in staining; +, barely visible staining; 0, no staining. Numbers are expressed as percents except for n, which denotes sample size.
RNA encoding β-galactosidase (200 pg), which was used as a lineage label, was injected, either alone or together with constitutively active or DnCaM KIV RNAs, into dorsal midline animal pole blastomeres of 32-cell stage embryos (A1 blastomeres by the nomenclature of Nakamura and Kishiyama, 1971; D1.1.1 by the nomenclature of Mills et al., 1999). These blastomeres give rise to anterior ectodermal derivatives and do not contribute to erythroid cells in the VBI (Mills et al., 1999). As a control, the same RNAs were injected into the two ventral midline animal pole cells (A4 blastomeres) of sibling 32-cell embryos. These blastomeres give rise to erythroid cells in a minor fraction of embryos (Mills et al., 1999). Half of the injected embryos in each group were stained for β-galactosidase activity at the tailbud stage (to verify the accuracy of injections), followed by in situ hybridization to detect globin transcripts (purple stain, black arrows). The rightmost image shows a Northern blot of globin expression in whole-control embryos or in recombinants of ventral mesoderm with ectoderm made to express mutant CaM kinases, as indicated above each lane. The blot was stripped and rehybridized with an EF-1α probe as a loading control. (C, left) Western blots of protein extracted from un.injected (control) embryos and embryos injected with plasmid expression vectors encoding mutant CaM kinases, as indicated. Endogenous CaM KK protein is readily detected throughout development, whereas ectopic CaM KKc protein (lower band) is first detected at stage 10. Endogenous CaM KIV protein is not detectable on the exposures shown. (C, right) RT-PCR analysis of globin expression in un.injected (control) embryos and embryos injected with plasmid expression vectors encoding mutant CaM kinases, as indicated above each lane.

above in Fig. 6 A, RNA encoding β-galactosidase (200 pg), which was used as a lineage label, was injected, either alone or together with constitutively active or DnCaM KIV RNAs, into dorsal midline animal pole blastomeres of 32-cell stage embryos (A1 blastomeres by the nomenclature of Nakamura and Kishiyama, 1971; D1.1.1 by the nomenclature of Mills et al., 1999). These blastomeres give rise to anterior ectodermal derivatives and do not contribute to erythroid cells in the VBI (Mills et al., 1999). As a control, the same RNAs were injected into the two ventral midline animal pole cells (A4 blastomeres) of sibling 32-cell embryos. These blastomeres give rise to erythroid cells in a minor fraction of embryos (Mills et al., 1999). Half of the injected embryos in each group were stained for β-galactosidase activity at the tailbud stage (to verify the accuracy of injections), followed by in situ hybridization to detect globin transcripts. RNA was extracted from the remaining embryos for quantitative analysis of globin expression by Northern blot hybridization. Misregulation of CaM KIV activity in the progeny of A1 blastomeres (indicated by red β-galactosidase staining, white arrows) led to a notable reduction in globin staining (black arrows) in many embryos (Fig. 6 A; Table II). Northern blot analysis revealed that when CaM KIV activity was misregulated in the progeny of A1 or A4 blastomeres, globin expression was reduced to 45–64% of control levels (Fig. 6 B). These data suggest that CaM KIV functions in nonhematopoietic cells to regulate production of a signal(s) that is required for normal erythropoiesis.

**Misregulation of CaM KIV after the Onset of Gastrulation Does Not Disrupt Hematopoiesis**

To examine the developmental period during which erythroid progenitors are sensitive to changes in CaM KIV activity, we analyzed expression of globin in ventral mesodermal explants that were cocultured with ectodermal cells in which CaM KIV activity was misregulated. As illustrated in Fig. 7 A, RNA encoding β-galactosidase (200 pg) was injected either alone or together with constitutively active or DnCaM KIV RNAs, near the animal pole of two-cell embryos. At the early gastrula stage (stage 10), ectoderm was isolated from injected embryos and cocultured with VMZ explants from un.injected sibling embryos until control tailbud stage 32. (B) Left three images show photographs of stage 32 ectoderm/mesoderm recombinants that were stained for β-galactosidase activity (red punctate stain) followed by in situ hybridization to detect globin transcripts (purple stain, black arrows). The rightmost image shows a Northern blot of globin expression in whole-control embryos or in recombinants of ventral mesoderm with ectoderm made to express mutant CaM kinases, as indicated above each lane.
cells in almost all cases, even when CaM KIV activity was misregulated in the ectodermal half (β-galactosidase positive cells are stained red) of the recombinant. Northern blot analysis of globin expression in the remaining recombinants confirmed that misregulation of CaM KIV in ectodermal cells beginning at the gastrula stage did not interfere with erythropoiesis (Fig. 7 B, rightmost image).

To further test the possibility that CaM KIV functions before gastrulation to regulate erythropoiesis, we used a DNA expression system to introduce dominant-negative or CaM KIVc into early gastrula-stage embryos. Unlike synthetic RNA, which is translated immediately after it is introduced into the embryo, injected DNA is first transcribed at midblastula (or later) stages, and transcripts are translated shortly thereafter. Plasmid DNA (200 pg) encoding constitutively active (pCS2-KIVc/KKc) or dominant-negative (pCS2-DnCaM KIV) CaM kinase under the control of a CMV promoter was injected near the VMZ of four-cell embryos. Western blot analysis of extracts from staged embryos revealed that ectopic CaM KIV and CaM KK proteins were first detectable at gastrula stage 10 (Fig. 7 C, left). Misregulation of CaM KIV activity beginning at the gastrula stage did not inhibit expression of globin in tailbud stage embryos as analyzed by RT-PCR (Fig. 7 C, right). Taken together, these data suggest that CaM KIV functions before the onset of gastrulation to regulate erythropoiesis.

**Misregulation of CaM KIV Does Not Disrupt Hematopoietic Specification**

The decrease in globin expression caused by either upregulation or inhibition of CaM KIV activity could be due to defects at any stage in RBC development, including specification of HSCs from ventral mesoderm, commitment of a subset of these pluripotent cells to the erythroid lineage, and/or proliferation, survival, or differentiation of hematopoietic precursors (Zon, 1995; Evans, 1997). To ask whether misregulation of CaM KIV interferes with the initial specification of HSCs, we analyzed expression of two early markers of blood development, Xaml (Tracey et al., 1998) and GATA-1 (Kelley et al., 1994). Neurula stage 18 embryos, in which CaM KIV was misregulated in ventral cells, expressed Xaml (Fig. 8 A) and GATA-1 (Fig. 8 B) at levels indistinguishable from control embryos. By contrast, at tailbud stage 28 expression of GATA-1 (Fig. 8 B) and Xaml (data not shown) was repressed in embryos injected with mutant CaM KIV RNAs. Since Xaml is initially expressed in cells derived from the dorsal side of embryos (Tracey et al., 1998), we also examined its expression following dorsal misregulation of CaM KIV and obtained identical results (data not shown). We conclude that misregulation of CaM KIV activity does not disrupt the initial specification of hematopoietic fate.

**Upregulation and Downregulation of CaM KIV Causes Distinct Hematopoietic Defects**

A decrease in globin expression is observed after either upregulation or inhibition of CaM KIV, but, as described below, we found that distinct mechanisms underlie this common embryonic phenotype. The inhibition of CaM KIV causes commitment of hematopoietic precursors to myeloid differentiation at the expense of erythroid differentiation, whereas constitutive activation of CaM KIV induces RBC progenitors to undergo apoptotic cell death.

To look for specific defects in hematopoietic maturation and differentiation, we examined RBC number and morphology in cytospin preparations of peripheral blood samples isolated from stage 45 tadpoles. At this stage of development, all of the circulating blood is derived from the VBI as a result of primitive hematopoiesis (Turpen et al., 1997). In control embryos, ~80% of circulating cells were RBCs and 20% were white blood cells (WBCs) (Fig. 9, A and B) that are predominantly of the monocyte/macrophage lineage (Ohnata et al., 1990). In embryos where CaM KIV activity was inhibited by injection of RNA encoding DnCaM KIV into either ventral (Fig. 9) or dorsal (data not shown) cells, the total number of circulating blood cells was unchanged, but a greater proportion of these were myeloid, as opposed to erythroid cells (Fig. 9, A and B) such that the ratio of WBCs to RBCs was increased approximately eightfold relative to controls (C). Based on morphology (Fig. 9 A) (Hadji-Azimi et al., 1987) and staining with alpha-naphthyl acetate esterase (data not shown), the majority of WBCs in these embryos, as in controls, were of the monocyte/macrophage lineage. In support of this conclusion, expression of myeloperoxidase, a marker of early embryonic macrophages (Smith, S., and T. Wayman et al. CaM KIV Regulates Primitive Hematopoiesis 819
Figure 9. Activation and inhibition of CaM KIV causes distinct defects in hematopoiesis. (A) Low (top row) and high (bottom row) magnification views of Wright-Giemsa–stained cytospin preparations of blood collected from control tadpoles or from tadpoles in which CaM KIV activity had been misregulated in ventral cells. Black arrows, WBCs; white arrows, abnormal RBCs. (B) The mean number (± SEM) of RBCs (black bars), WBCs (open bars), and total blood cells (shaded bars) present in three random fields of cytospin blood preparations from control or experimental embryos are shown. At least 100 embryos from three independent experiments were averaged for each point. (C) The ratio of WBCs to RBCs were calculated from the data shown in B. (D) Northern blot analysis of myeloperoxidase expression in tadpole stage 41 em-
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Erythroid Lineage

During embryogenesis, primitive blood progenitors are committed almost exclusively to erythroid differentiation (Tavassoli, 1991), yet can be induced to adopt a myeloid or lymphoid fate when cultured in the presence of appropriate hematopoietic differentiation factors in vitro (Moore and Metcalf, 1970), or when transplanted to a different hematopoietic site in vivo (Huang and Auerbach, 1993; Turpen et al., 1997). These studies suggest that primitive blood progenitors are pluripotent, but that factors present in their microenvironment restrict their fate and direct them towards erythroid differentiation. Signals that function non-cell-autonomously to restrict the developmental potential of primitive blood progenitors have not been identified. Our studies involving inhibition of endogenous CaM KIV demonstrate that CaM KIV is required to generate these signals.

The increase in WBC number in CaM KIV–deficient embryos could potentially result from expansion of a population of progenitors that arise independent of the VBI, rather than from commitment of primitive HSCs to myeloid differentiation, as proposed above. Grafting experiments in Xenopus have demonstrated the existence of non-lymphoid leukocytes (primarily macrophages) that are not derived from primitive stem cells of the VBI, but originate entirely from the head region of the embryo (Ohinata et al., 1990). More recent studies in zebrafish have identified a similar population of self-renewing macrophage precursors that are not derived from the blood islands (Herbomel et al., 1999). At present, we cannot rule out the possibility that inactivation of CaM KIV induces the proliferation of this distinct leukocyte population. However, this seems unlikely given that we do not see an increase in myeloperoxidase-positive cells in CaM KIV–deficient embryos before formation of the VBI (data not shown). Furthermore, the observation that the increase in WBCs in CaM KIV–deficient embryos is accompanied by a corresponding decrease in RBCs is more consistent with the hypothesis that VBI-derived stem cells become committed to myeloid differentiation at the expense of the erythroid lineage.

Downregulation of CaM KIV Activity May Be Required for Survival and Maturation of Erythroid Progenitors

Our studies show that though a certain level of CaM KIV activity is required for commitment of HSCs to erythroid differentiation, abnormally high levels of CaM KIV result in apoptosis of erythroid progenitors. Programmed cell death is a major component of normal erythropoiesis (Koury and Bondurant, 1990) and provides a mechanism to upregulate or downregulate red cell number in response to environmental conditions. This process has been best characterized for erythroid progenitors of definitive hematopoietic stem cells in which CaM KIV activity was misregulated. Ethidium bromide staining of the RNA gel before transfer is shown as a loading control. (E) Nuclei of cells were stained with propidium iodide. Arrows indicate TUNEL-positive cells. (F) The percent of blood cells that are apoptotic in three random fields of cytospin blood preparations from at least 24 embryos. The data presented are the mean ± SEM.
Apoptosis. The survival, proliferation, and differentiation of these cells is highly dependent on the hormone erythropoietin (EPO) (for review see Krantz, 1991), which exerts its antiapoptotic effects, at least in part, by inducing expression of Bcl-x (Socolovsky et al., 1999). Programmed cell death most likely also plays an important role in regulating the production of primitive erythrocytes, since mice lacking either EPO or its receptor show partial defects in yolk sac hematopoiesis (Wu et al., 1995). Furthermore, Bcl-x appears to be required for the survival of primitive, as well as definitive, erythrocytes (Motoyama et al., 1999), though this remains controversial (Gregory et al., 1999). It is possible that inappropriate activation of CaM KIV inhibits expression of EPO or another antiapoptotic hormone, thereby leading to excessive death of primitive RBCs.

A caveat of our analysis of phenotypic defects caused by hyperactivation of the CaM KIV–signaling pathway is that these studies can only demonstrate potential functions for this enzyme. Further studies will be needed to determine whether negative regulation of endogenous CaM KIV signaling is required to prevent apoptosis during the normal program of erythroid maturation.

CaM KIV Regulates Production of Extrinsic Signals That Direct Normal Hematopoiesis

Based on the well-documented role of CaM KIV in modulating transcription factor function, a simple hypothesis is that the hematopoietic defects caused by misregulation of CaM KIV activity are a direct result of changes in transcriptional regulation of the blood development program. Numerous hematopoietic-specific transcription factors that participate in proliferation, lineage commitment, survival, and/or differentiation of HSCs have been identified that might be regulated by CaM KIV. For example, GATA-1, SCL, and LMO-2 have been implicated as transcriptional regulators that are required for commitment of progenitors to the erythroid lineage (for review see Sieweke and Graf, 1998), whereas the Ets family member PU.1 can instruct pluripotent hematopoietic progenitors to differentiate along the myeloid lineage (Nerlov and Graf, 1998). GATA-1 is also required to block apoptosis and thus permit survival and maturation of erythroid precursors (Weiss and Orkin, 1995; De Maria et al., 1999). CaM KIV could potentially function cell autonomously within HSCs to activate or inactivate one or more of these transcriptional regulators, either directly, by phosphorylating the factor itself, or indirectly, by regulating the activity of cofactors such as cAMP-responsive element-binding protein–(CREB) binding protein (Hung et al., 1999).

As described below, our data demonstrate that CaM KIV regulates the production of extrinsic signals that act upstream of blood cell–specific transcription factors to control hematopoiesis. Unfortunately, this non-cell–autonomous precludes the use of the Xenopus embryo system to definitively test whether CaM KIV directly regulates signals intrinsic to blood progenitors. Further analyses of CaM KIV function in purified populations of HSCs, or in transgenic mice in which CaM KIV is specifically misregulated in HSCs, will help to resolve this issue.

The observation that erythropoiesis is defective even when CaM KIV activity is perturbed solely in nonerythroid tissues demonstrates a non-cell–autonomous role for this enzyme. Embryologic studies have shown that signals produced by nonhematopoietic tissues are required for normal blood development. In Xenopus, explant studies have shown that signals from the dorsal lateral plate can stimulate progenitors in the VBI to differentiate as myeloid cells of the monocyte/macrophage lineage (Turpen and Smith, 1985). Tissue recombination studies have further shown that signals from ectodermal cells, including those in the dorsal half of the animal pole, are required for erythroid cells to synthesize globin protein (Maeno et al., 1994). Finally, transplantation studies have shown that signals present in ventral regions of the embryo are required for commitment of multipotential progenitors to primitive hematopoiesis during gastrulation, and they are sufficient to induce cells that would normally contribute only to definitive hematopoiesis to, instead, form primitive erythrocytes (Turpen et al., 1997). Extrinsic signals are also required for mammalian hematopoiesis. For example, signals generated by the primitive endoderm are required for the initial induction of mesoderm with hematopoietic potential (Belaoussoff et al., 1998). Furthermore, factors produced by endothelial or endodermal-like cell lines derived from the yolk sac (Yoder et al., 1994; Fennie et al., 1995), or other hematopoietic sites (Ohneda et al., 1998), can stimulate self-renewal and direct the lineage commitment and maturation of primitive and definitive HSCs. One hypothesis that is consistent with our results is that endogenous CaM KIV regulates expression of a gene(s) encoding a cell–cell signaling molecule(s) that normally functions to promote survival/proliferation of primitive erythrocytes, but which can specify HSCs to adopt a myeloid fate when present at abnormal levels. This hypothesis could account for the observation that both upregulation and inhibition of CaM KIV activity cause defects in hematopoiesis, since both would lead to abnormal levels of this gene product.

Despite embryological evidence showing that cell–cell signaling between HSCs and their environment is required for normal blood development, the molecular nature of the extrinsic signals that dictate the fate of blood progenitors is unknown. Mutations in many unidentified genes have been shown to disrupt various aspects of hematopoiesis in zebrfish (for review see Parker et al., 1999), and at least one of these (cloche) functions non–cell–autonomously (Parker and Stainier, 1999). Our studies demonstrate a critical role for CaM KIV in the generation of extrinsic signals that regulate several different steps in erythroid development. Further analysis of when and where CaM KIV functions during embryonic hematopoiesis will provide a first step toward the eventual identification of extrinsic signals that control the blood program.

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References

Ahn, S., D.D. Ginty, and D.J. Linden. 1999. A late phase of cerebellar long-term depression requires activation of CaMIV and CREB. 

Anderson, K.A., T.J. Ribar, M. Illario, and A.R. Means. 1997. Defective survival and activation of thymocytes in transgenic mice expressing a catalytically inactive form of Ca2+/calmodulin-dependent protein kinase IV. 

Belousoff, M., S.M. Farrington, and M.H. Baron. 1998. Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. 

Bhatia, M., D. Fozard, W. Wu, B. Murdoch, J. Wrana, L. Gallacher, and J.E. Dick. 1999. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. J. Exp. Med. 189:1139–1148.

Bito, H., K. Deisseroth, and R.W. Tsien. 1996. CREB phosphorylation and dephosphorylation: a Ca2+/and stimulus duration-dependent switch for hippocampal gene expression. Cell. 87:1203–1214.

Christian, J.L., and R.T. Moon. 1993. Interactions between Xwnt-8 and Six-1. 

Enslen, H., H. Tokumitsu, P.J. Stork, R.J. Davis, and T.R. Soderling. 1996. Bito, H., K. Deisseroth, and R.W. Tsien. 1996. CREB phosphorylation and dephosphorylation: a Ca2+/and stimulus duration-dependent switch for hippocampal gene expression. Cell. 87:1203–1214.

Christian, J.L., and R.T. Moon. 1993. Interactions between Xwnt-8 and Spe.

Fredieu, R.J., Y. Cui, D. Maier, M.V. Danilchik, and J.L. Christian. 1997. Aberphos and differentiation of hematopoietic progenitors. Blood. 86:4454–4467.

Herbomel, P., B. Thiese, and C. Thiese. 1999. Ontogeny and behavior of early hematopoietic stem cells from the yolk sac of in vivo and in vitro colony forming cells in the developing mouse embryo. Br. J. Haematol. 108:272–286.

Wayman et al. 

Ahmed, S.M. Srinivasula, E.S. Alnemri, U. Testa, and C. Peschle. 1999. Negative regulation of neuronal nitric-oxide synthase by calmodulin protein kinase IV. Mol. Cell. Biol. 110:5039–5048.

Wayman et al. 

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Wayman et al. 

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Wayman et al. 

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Wayman et al. 

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Wayman et al. 

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Wayman et al. 

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Wayman et al. 

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Wayman et al. 

Ahmed, S.M. Srinivasula, E.S. Alnemri, U. Testa, and C. Peschle. 1999. Negative regulation of neuronal nitric-oxide synthase by calmodulin protein kinase IV. Mol. Cell. Biol. 110:5039–5048.
tral lateral plate mesoderm during early development of *Xenopus laevis* embryos. *J. Leukoc. Biol.*, 38:415–427.

Turpen, J.B., C.M. Kelley, P.E. Mead, and L.I. Zon. 1997. Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity*, 7:325–334.

Wayman, G.A., J. Wei, S. Wong, and D.R. Storm. 1996. Regulation of type I adenylyl cyclase by calmodulin kinase IV in vivo. *Mol. Cell. Biol.*, 16:6075–6082.

Wayman, G.A., H. Tokumitsu, and T.R. Soderling. 1997. Inhibitory cross-talk by cAMP kinase on the calmodulin-dependent protein kinase cascade. *J. Biol. Chem.*, 272:16073–16076.

Weber, R., B. Blum, and P.R. Muller. 1991. The switch from larval to adult globin gene expression in *Xenopus laevis* is mediated by erythroid cells from distinct compartments. *Development*, 112:1021–1029.

Weiss, M.J., and S.H. Orkin. 1995. Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. *Proc. Natl. Acad. Sci. USA.*, 92:9623–9627.

Wu, H., X. Liu, R. Jaenisch, and H.F. Lodish. 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell*, 83:59–67.

Yano, S., H. Tokumitsu, and T.R. Soderling. 1998. Calcium promotes cell survival through CaM-K kinase activation of the protein kinase-B pathway. *Nature*, 396:584–587.

Yoder, M.C., V.E. Papaioannou, P.P. Breitfeld, and D.A. Williams. 1994. Murine yolk sac endoderm- and mesoderm-derived cell lines support in vitro growth and differentiation of hematopoietic cells. *Blood*, 83:2438–2443.

Zon, L.I. 1995. Developmental biology of hematopoiesis. *Blood*, 86:2876–2891.

Zon, L.I., C. Mather, S. Burgess, M.E. Bolce, R.M. Harland, and S.H. Orkin. 1991. Expression of GATA-binding proteins during embryonic development in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA.*, 88:10642–10646.