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A novel crosstalk between calcium/calmodulin kinases II and IV regulates cell proliferation in myeloid leukemia cells

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ABSTRACT

CaMKs link transient increases in intracellular Ca^{2+} with biological processes. In myeloid leukemia cells, CaMKII, activated by the *bcr-abl* oncogene, promotes cell proliferation. Inhibition of CaMKII activity restricts cell proliferation, and correlates with growth arrest and differentiation. The mechanism by which the inhibition of CaMKII results in growth arrest and differentiation in myeloid leukemia cells is still unknown. We report that inhibition of CaMKII activity results in an upregulation of CaMKIV mRNA and protein in leukemia cell lines. Conversely, expression of CaMKIV inhibits autophosphorylation and activation of CaMKII, and elicits G_0/G_1cell cycle arrest, impairing cell proliferation. Furthermore, U937 cells expressing CaMKIV show elevated levels of Cdk inhibitors $p27^{kip1}$ and $p16^{ink4a}$ and reduced levels of cyclins A, B_1 and D_1.
These findings were also confirmed in the K562 leukemic cell line.
The relationship between CaMKII and CaMKIV is also observed in primary acute myeloid leukemia (AML) cells, and it correlates with their immunophenotypic profile. Indeed, immature MO/M1 AML showed increased CaMKIV expression and decreased pCaMKII, whereas highly differentiated M4/M5 AML showed decreased CaMKIV expression and increased pCaMKII levels.
Our data reveal a novel cross-talk between CaMKII and CaMKIV and suggest that CaMKII suppresses the expression of CaMKIV to promote leukemia cell proliferation.

1. Introduction

Multifunctional CaMKs (CaMKI, CaMKII and CaMKIV) are important mediators of intracellular Ca^{2+} signalling that play pleiotropic roles in cell physiology. These serine–threonine (Ser/Thr) protein kinases are activated upon Ca^{2+}/CaM binding. The upstream CaMK kinases (CaMKKs) phosphorylate a critical Thr 200 in the activation-loop to activate CaMKIV, whereas CaMKII is fully activated by autophosphorylation of its own Thr 286 [1,2]. Ca^{2+}/CaM signaling is necessary for cell cycle progression, and CaM-dependent pathways influence cyclin-Cdk activation at different phases of the cell cycle [3]. Several studies suggest the involvement of CaMKs at G_1/S and G_2/M transitions of the cell cycle [4–8]. Furthermore, treatment of several cell types with KN93, a pharmacological inhibitor of CaMKs, elicits a reversible G_1 arrest that correlates with enhanced association of $p27^{kip1}$ with Cdk2 and reduced Cdk2/4 activity [9].

CaMKIV is a predominantly nuclear, monomeric kinase with tissue-restricted expression [1]. It plays a role in mediating Ca^{2+}-regulated transcription through phosphorylation and/or activation of different transcription factors, including cyclic adenosine mono phosphate

Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}-calmodulin dependent kinase; RAR, retinoic acid receptor; CDKi, cyclin dependent kinase inhibitor; AML, acute myeloid leukemia; Rb, retinoblastoma.

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(cAMP) response element binding protein (CREB), activating transcription factor (ATF), serum response factor (SRF), and transcriptional co-factors including CREB binding protein (CBP) [10]. Embryonic expression of CaMKIV correlates with periods of differentiation and terminal mitoses, supporting for this kinase a pivotal role in regulation of cell differentiation [11]. Indeed, in hematopoietic stem cells (HSCs), CaMKIV/CREB/CBP pathway restricts cell proliferation by expression of Bcl-2, a pro-survival protein with additional roles in cell quiescence [12]. In cancer cells, CaMKs appear to play a role in aggressiveness: CaMKK2 signaling through adenosine monophosphate dependent protein kinase (AMPK) [13] and/or CaMKI [3] modulates cell migration, invasiveness and malignancy in prostate cancer and medulloblastoma [14,15]. Notably, CaMKIV is not expressed in these two cancer cell types, a feature that the authors attributed to reduced expression of genes required for differentiation [14].

The ubiquitously expressed, multimeric CaMKII is predominantly cytoplasmic, although 3 alternatively spliced variants: α_B,γ_A, and δ_B are targeted to the nucleus [16,17]. CaMKII exerts a broad range of biological functions, and is an important regulator of cell proliferation [18,19]. It modulates Raf1 activity leading to extra-cellular receptor kinase (ERK) activation and induction of cell proliferation. The interplay between CaMKII and ERK has been observed in several cell types including thyroid [20,21], fibroblasts [22], skeletal muscle [23] and vascular smooth muscle [24], suggesting that it is a general mechanism in the control of cell proliferation. CaMKII is also a target of RET-PTC oncogene, raising the possibility that it plays a central role in the abnormal growth of tumor cells [25].

Many studies suggest the involvement of CaMKII in the homeostasis of tumor cells. Indeed, Si and Collins reported that myeloid leukemia cell lines as well as primary acute myeloid leukemia (AML) patient samples express elevated levels of activated CaMKII. CaMKII promotes G_1/S cell cycle progression [26] and its inhibition impairs myeloid leukemia cell proliferation. Leukemia cells undergoing growth arrest and/or terminal differentiation present markedly reduced levels of CaMKII [26], suggesting that downregulation of CaMKII may be a pre-requisite for inducing terminal differentiation in myeloid leukemia cells.

Because of the reciprocal aspects of CaMKII and CaMKIV on proliferation [12] and terminal differentiation [11,27,28] in hematopoietic progenitors, we hypothesized that CaMKIV may be repressed in a CaMKII dependent manner in myeloid leukemia cells. In the present study, we investigated a potential cross-talk between CaMKII and CaMKIV in human myeloid leukemia U937 cells [29], which express very low CaMKIV and high CaMKII levels. We found that CaMKII represses *CaMK4* transcription and promotes proliferation in human myeloid leukemia cells. On the other hand, transgenic enhanced expression of CaMKIV negatively modulates CaMKII activity and induces G_0/G_1 cell cycle arrest in these malignant cells. We propose that CaMKII and CaMKIV counterregulation is not restricted to leukemia cells and can be exploited in the development of targeted therapies against myeloid leukemia and other myeloid disorders.

2. Materials and methods

2.1. Cell culture and reagents

U937 and K562 (CRL1593.2 and CCl243, respectively, American Type Culture Collection (ATCC), Manassas, VA) were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Human embryonic kidney HEK 293A cells (R70507, Invitrogen) were grown in DMEM supplemented with 10% FBS (Invitrogen). Cells were stimulated with 2 μM ionomycin (Sigma, St. Louis, MO) for 15 min or with 10% FBS following 12 h serum starvation. CaMKII pharmacological inhibition was obtained with 10 μM of the CaMKs inhibitor KN93 (Biomol Plymouth Meeting, PA); or with 5 μM of the CaMKII selective inhibitory peptide, AntCaNtide [30].

2.2. RNA interference

Four CaMKIIγ-specific shRNA (SureSilencing shRNA plasmids) and control shRNA vector were purchased from SuperArray. The targeted sequences are shRNA1: ACCTGCTGCTGGCGAGTAAAT; shRNA2;GAACGTGA GGCTCGGATATGT; shRNA3: GAGGCCTACACGAAGATTTGT; shRNA-4: GAGTGTTTGCGCAAGTTCAAT. CaMKIIγ-shRNA design was based on the GenBank accession number NM_001222. CaMKIIγ- shRNA transfections were performed using Attractene Transfection Reagent.

These four plasmids were, first, transiently transfected in U937 cells according to the manufacturer's instructions, and then selected in G418 (800 μg/mL) for 10 d. We first indentified one shRNA that caused >80% reduction in the endogenous CaMKIIγ levels.

2.3. Lentiviral infection

Lentivirus-green fluorescent protein (GFP) constructs were generated and characterized as mentioned before [31]. Briefly, full-length WT CaMKIVcDNA (a gift from Dr. Anthony R. Means, Duke University Medical Center) was cloned into a Lenti-green fluorescent protein (GFP) vector [32]. Empty Lenti-GFP-control ("Mock"), Lenti-GFP-CaMKIV ("CaMKIV") and Lenti-GFP-CaMKIV-K71M viruses were generated as previously described [3,4] using second generation helper plasmids in 293T cells (a gift from Dr. Anthony R. Means, Duke University Medical Center and described in Kitsos et al. [12]). Virus titers were calculated using 293T cells and CaMKIV expression confirmed by immunoblotting. Approximately 2×10^6 of U937 cells were infected with control (Mock) or Lenti-GFP-CaMKIV (CaMKIV) viruses at a multiplicity of infection of 10:1.

2.4. Cell viability and [^{3}H] thymidine (T) incorporation assays

50,000 cells/well were counted daily following trypan blue staining (Invitrogen). 5000 cells were serum-starved for 12 h, and DMEM-10% FBS with 0.5 μCi [^{3}H] thymidine (T) and CaMKII inhibitors were added. After 24 h, [^{3}H] T uptake was evaluated (BD Biosciences, San Jose, CA).

2.5. Cell cycle distribution assay

Cells were fixed in 70% ethanol, incubated with 0.02 mg/ml propidium iodide (PI) and 0.25 mg/ml ribonuclease A (Sigma), and analyzed for DNA content (FACScan, BD). Acquisition and analyses were performed using CellQuest (BD) software.

2.6. Annexin V apoptosis assay

3×10^6cells were incubated with AnnexinV-PE and PI (BDPharmingen, USA) and analyzed by flow cytometry. Samples were acquired with a CYAN flow cytometer (DAKO Corporation, San Jose, CA) and analyzed using SUMMIT® software.

2.7. Ki-67 staining

Cells were incubated with Ki-67-PE antibody and 7-amino actinomycin D (7AAD, BD) and analyzed (FACSAria, BD).

2.8. Bromo deoxy uridine (BrdU) labeling

Cells were pulse-labeled with 10 μM BrdU (Sigma). Samples were processed and incubated with BrdU-PE antibody and 7AAD (BD), and analyzed on a FACSAria.