



# CaMKII regulates retinoic acid receptor transcriptional activity and the differentiation of myeloid leukemia cells

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**Retinoic acid receptors (RARs) are members of the nuclear hormone receptor family and regulate the proliferation and differentiation of multiple different cell types, including promyelocytic leukemia cells. Here we describe a biochemical/functional interaction between the Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs) and RARs that modulates the differentiation of myeloid leukemia cells. We observe that CaMKII $\gamma$  is the CaMK that is predominantly expressed in myeloid cells. CaMKII inhibits RAR transcriptional activity, and this enzyme directly interacts with RAR through a CaMKII LxxLL binding motif. CaMKII $\gamma$  phosphorylates RAR $\alpha$  both in vitro and in vivo, and this phosphorylation inhibits RAR $\alpha$  activity by enhancing its interaction with transcriptional corepressors. In myeloid cell lines, CaMKII $\gamma$  localizes to RAR target sites within myeloid gene promoters but dissociates from the promoter upon retinoic acid-induced myeloid cell differentiation. KN62, a pharmacological inhibitor of the CaMKs, enhances the terminal differentiation of myeloid leukemia cell lines, and this is associated with a reduction in activated (autophosphorylated) CaMKII in the terminally differentiating cells. These observations reveal a significant cross-talk between Ca<sup>2+</sup> and retinoic acid signaling pathways that regulates the differentiation of myeloid leukemia cells, and they suggest that CaMKII $\gamma$  may provide a new therapeutic target for the treatment of certain human myeloid leukemias.**

## Introduction

Hematopoiesis involves an intricate, functional interaction between lineage-specific cytokines and lineage-specific transcription factors. Among these transcription factors the retinoic acid receptors (RARs) are important regulators of myeloid lineage differentiation. These receptors are members of the ligand-activated nuclear receptor family and include 2 distinct families, the RARs and retinoid X receptors (RXRs), which bind as RAR/RXR heterodimers to their specific target sequences, the retinoic acid response elements (RAREs). RARs play a critical role in regulating myeloid differentiation, since retinoic acid (RA) stimulates the granulocytic differentiation of normal hematopoietic precursors (1), and knockout mice deficient in both RAR $\alpha$  and RAR $\gamma$  exhibit a block to granulocyte differentiation (2). Moreover, a critical genetic event in the development of human acute promyelocytic leukemia (APL) involves the acquisition of a t(15;17) chromosome translocation resulting in the leukemogenic PML-RAR $\alpha$  fusion protein that acts as a dominant-negative RAR to block normal myeloid differentiation (3, 4). This differentiation block can be overcome with relatively high, pharmacological concentrations of RA, and the clinical use of RA to induce terminal differentiation of leukemia cells has had a remarkably beneficial impact on the therapy of APL (5, 6).

Ligand binding to RAR/RXR alters its transcriptional activity by triggering a conformational change in this complex that inhibits its interaction with transcriptional corepressors while enhancing its interaction with different transcriptional coactivators (7, 8). A signature LxxLL binding motif or closely related sequences are pres-

ent in the receptor interaction domains of a number of different nuclear hormone receptor coactivators, including the SRC/p160 family (9), the ASC-2 coactivator (10), and the mediator complex TRAP220/DRIP205/MED1 subunit (11, 12). This motif mediates the recruitment of these coactivators into the nuclear receptor transcription complex by directly contacting the AF-2 domain of the nuclear receptors. The transcriptional activity of the RARs is also regulated by protein kinase-mediated phosphorylation. Both protein kinase A and cdk7 phosphorylate and enhance RAR transcriptional activity (13, 14), and diminished cdk7 phosphorylation of RAR with reduced RAR activity is observed in the genetic disorder xeroderma pigmentosum (15).

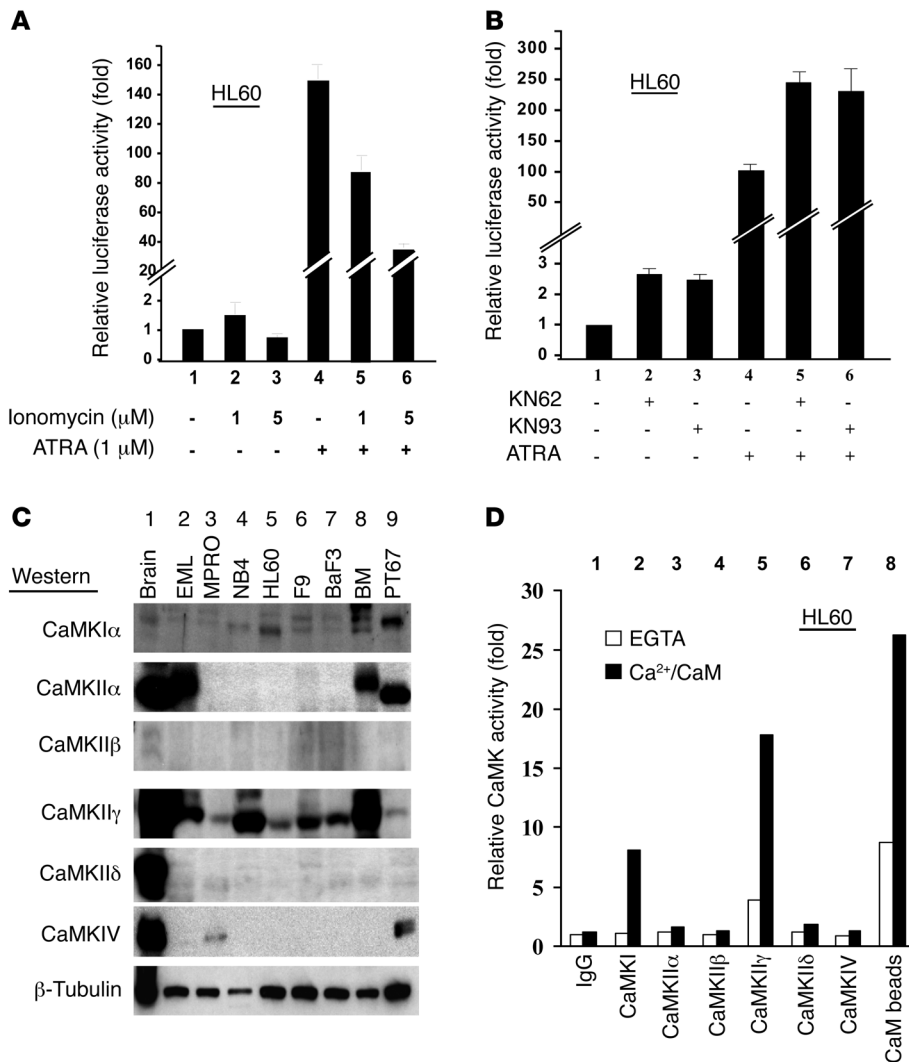
The Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs) are multifunctional serine/threonine kinases whose activity is regulated through Ca<sup>2+</sup> signaling (16). The most widely studied CaMKs include CaMKI, CaMKII, and CaMKIV. In general, CaMK activation is triggered by the binding of Ca<sup>2+</sup>/calmodulin (Ca<sup>2+</sup>/CaM), and the levels of Ca<sup>2+</sup>/CaM are regulated by changes in intracellular Ca<sup>2+</sup> concentration. The CaMKs regulate the development and activity of multiple different cell types. For example, CaMKII and CaMKIV regulate cytokine expression in activated T lymphocytes (17, 18). CaMKII also regulates dendritic morphogenesis through phosphorylation of the NeuroD transcription factor (19). Moreover, CaMKII, which constitutes approximately 1%–2% of total brain protein, also phosphorylates proteins involved in strengthening synaptic transmission, and this likely regulates learning and memory (reviewed in ref. 20).

In the present study we have explored the activity of the CaMKs in hematopoiesis and observe that CaMKII $\gamma$  is intimately involved in the RAR-mediated regulation of myelopoiesis. In myeloid cells we observe a direct physical and functional interaction of CaMKII $\gamma$  with RARs that is mediated through a CaMKII LxxLL signature motif. CaMKII $\gamma$  localizes to RAR target sites within myeloid gene promoters, and this enzyme phosphorylates RAR and inhibits its transcrip-

**Nonstandard abbreviations used:** APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; Ca<sup>2+</sup>/CaM, Ca<sup>2+</sup>/calmodulin; CaMK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase; C/EBP $\epsilon$ , CCAAT/enhancer binding protein- $\epsilon$ ; CHIP, chromatin immunoprecipitation; GST, glutathione S-transferase; RA, retinoic acid; RAR, RA receptor; RARE, RA response element.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Citation for this article:** *J. Clin. Invest.* 117:1412–1421 (2007). doi:10.1172/JCI30779.



**Figure 1**

CaMK isoform expression in hematopoietic cells. (A) The activity of an ATRA-responsive luciferase reporter (βRARE-tk-Luc, 25 μg) was assessed in electroporated HL60 cells 6 hours after treatment with the indicated concentrations of ionomycin and ATRA. (B) The activity of the luciferase reporter (βRARE-tk-Luc, 25 μg) was assessed in HL60 cells 6 hours after treatment with KN62 (5 μM), KN93 (5 μM), and ATRA (1 μM). (C) Western blots were performed using the indicated antibodies. F9 (lane 6) is a mouse embryonal carcinoma cell line. BaF3 (lane 7) is a cultured pre-B cell line. BM cells (lane 8) are kit ligand-dependent normal murine hematopoietic precursors. PT67 murine fibroblasts individually transduced with rat CaMKIIα, human CaMKIα, or human CaMKIV (lane 9) together with brain lysates (lane 1) serve as positive controls for the respective antibodies. (D) CaMK assays were performed on HL60 cell lysates immunoprecipitated with the indicated antibodies.

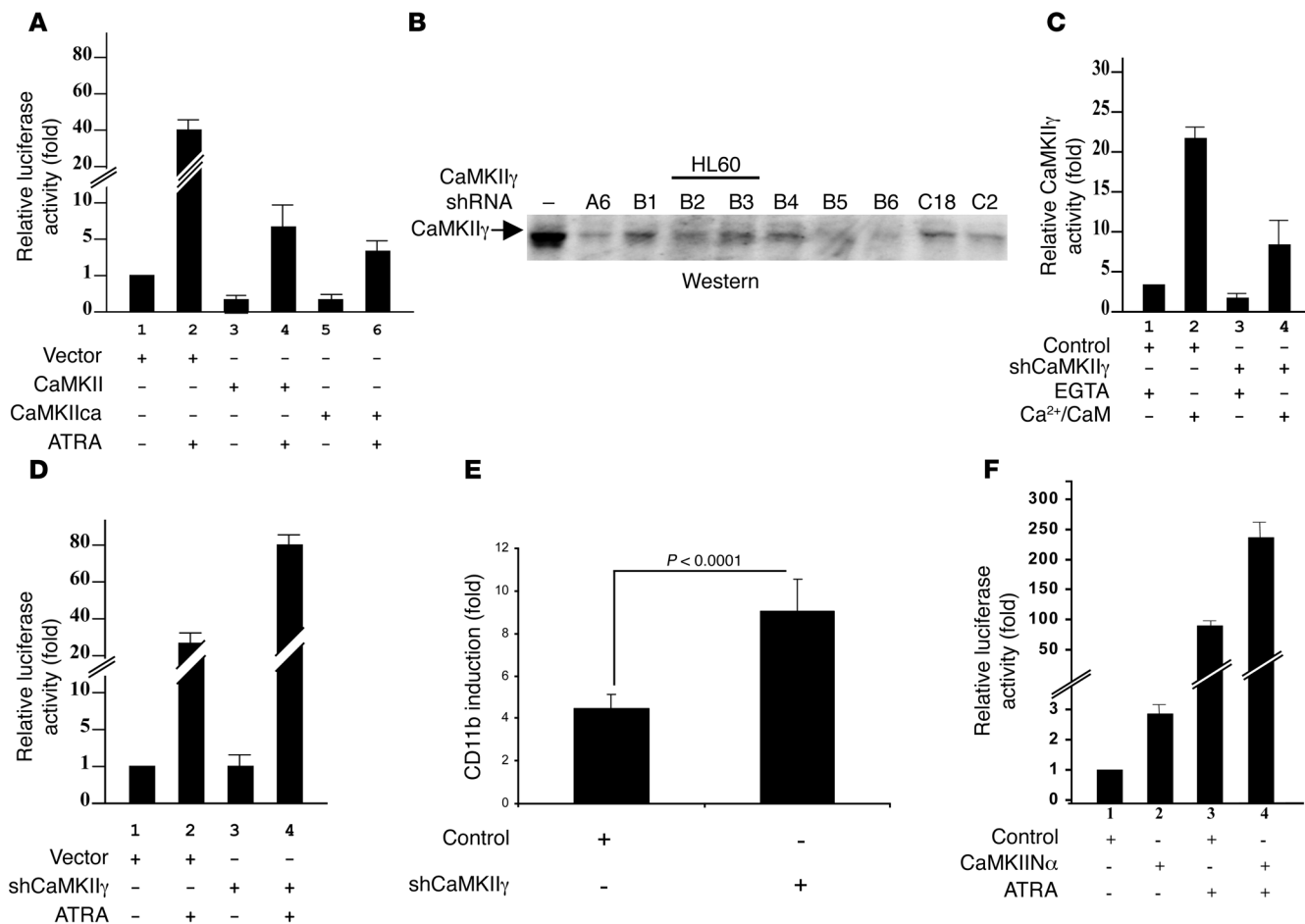
preparational activity. Pharmacological inhibitors of CaMKII markedly enhance the granulocytic differentiation of APL cells. These studies reveal a previously unexplored molecular interaction and cross-talk between Ca<sup>2+</sup> and RAR signal transduction pathways and suggest that CaMKIIγ may provide a new molecular target for the therapy of leukemia, particularly APL.

**Results**

*Expression and activity of the CaMKs in differentiating myeloid cells.* All-trans retinoic acid (ATRA) induces the terminal granulocytic differentiation of certain cultured hematopoietic cells, and we

have been using such cell lines, including HL60 (21), NB4 (22), and MPRO (23), to define factors that regulate RAR transcriptional activity in differentiating myeloid cells. We observed that the Ca<sup>2+</sup> ionophore ionomycin inhibited the ATRA-induced activity of a RARE-driven luciferase reporter in HL60 myeloid cells (Figure 1A, lanes 4–6). Such Ca<sup>2+</sup> ionophores can potentially regulate many different Ca<sup>2+</sup>-dependent enzymes, including the CaMKs. To assess whether the CaMKs are involved in regulating RAR transcriptional activity, we used KN62 and KN93, both potent small-molecule inhibitors of the CaMKs (IC<sub>50</sub> = 0.9 μM), which directly interfere with the binding of Ca<sup>2+</sup>/CaM to the different CaMKs (24, 25). We found that in HL60 myeloid cells, both KN62 and KN93 enhanced RAR transcriptional activity (Figure 1B). We also found similar results in other transfected myeloid cells, including MPRO and NB4 (not shown). The observed enhancement of RAR activity by small-molecule inhibitors of the CaMKs suggests that endogenous CaMKs are involved in downregulating the activity of the RARs in myeloid cells.

To further assess the role that the endogenous CaMKs might play in RAR activity and myeloid differentiation, we performed Western blots to compare the expression of the different CaMKs in different hematopoietic cells. We found low but detectable expression of CaMKI (CaMKIα) in all of the different hematopoietic cell types tested (Figure 1C, first row). CaMKII consists of 4 distinct but highly homologous genes (α, β, γ, and δ), and all of the hematopoietic cells displayed readily detectable levels of the CaMKIIγ isoform, while CaMKIIα expression was evident only in the immature, multipotent EML hematopoietic cells (26) and in normal immature bone marrow precursors (Figure 1C, lanes 2 and 8). In contrast with CaMKI and CaMKII, CaMKIV, while expressed in brain extracts (Figure 1C, lane 1) in the Jurkat T lymphoid cell line (not shown) and to a slight degree in MPRO cells (Figure 1C, lane 3), was not readily detected in any other hematopoietic cells. These Western blot observations were confirmed by RT-PCR with RNAs from these different cell types, which also indicated that CaMKIα and CaMKIIγ were the predominant CaMK mRNAs expressed by the NB4 and HL60 cell lines (not shown). We also quantitated both autonomous (Ca<sup>2+</sup>/CaM-independent) and total (Ca<sup>2+</sup>/CaM-dependent) CaMK enzyme activity in HL60 cell lysates following immunoprecipitation with different CaMK



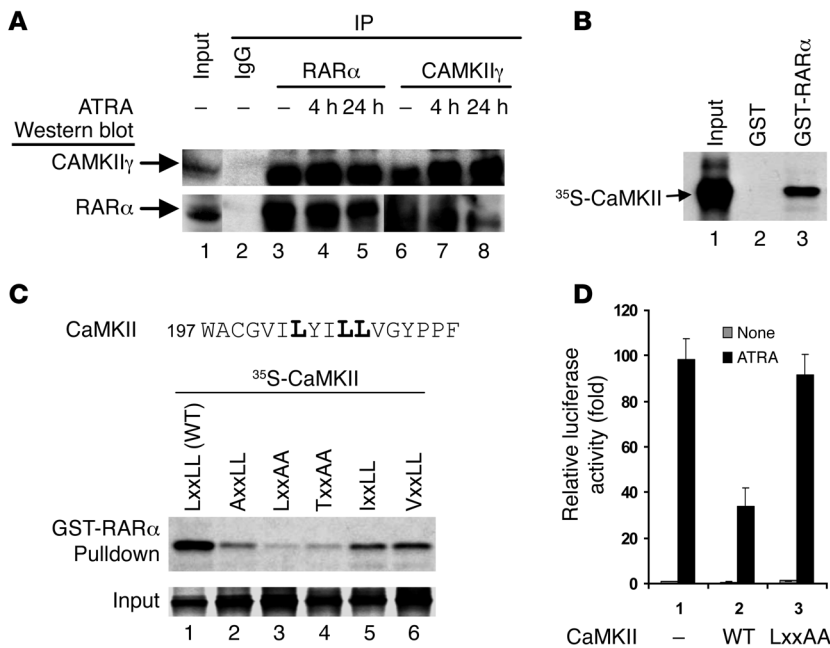
**Figure 2**

Regulation of RAR activity by CaMKII. **(A)** HL60 cells were electroporated with the  $\beta$ RARE-tk-Luc reporter (25  $\mu$ g) together with expression vectors (20  $\mu$ g) harboring WT or constitutively activated (ca) CaMKII cDNAs (CaMKII $\alpha$ ). ATRA (1  $\mu$ M) was added, and relative luciferase activity determined after 6 hours. **(B)** HL60 cells were stably transduced with empty (control) retroviral vectors and with vectors harboring CaMKII $\gamma$  shRNAs as detailed in Methods. Western blotting identified 6 CaMKII $\gamma$  shRNA-transduced HL60 subclones (A6, B2, B5, B6, C2, and C18) with reduced CaMKII $\gamma$  protein expression. **(C)** Pooled subclones of the CaMKII $\gamma$  shRNA vector-transduced HL60 cells that exhibited reduced CaMKII $\gamma$  expression on Western blots **(B)** together with control (empty) vector-transduced HL60 cells were lysed and immunoprecipitated with CaMKII $\gamma$  antibody; the immunoprecipitates were assayed for both Ca<sup>2+</sup>/CaM-independent (EGTA) and Ca<sup>2+</sup>/CaM-dependent (Ca<sup>2+</sup>/CaM) enzyme activity. **(D)** Pooled subclones described in **C** that exhibited reduced CaMKII $\gamma$  expression/activity **(B and C)** as well as control (empty) vector-transduced cells were electroporated with the  $\beta$ RARE-tk-Luc reporter (25  $\mu$ g), and relative luciferase activity was determined as described above. **(E)** The subclones described in **C** that exhibited reduced CaMKII $\gamma$  expression/activity **(B and C)** as well as control vector-transduced cells were treated with RA (1 nM) for 5 days followed by FACS quantitation of Cd11b surface antigen. **(F)** HL60 cells were electroporated with the  $\beta$ RARE-tk-Luc reporter (25  $\mu$ g) together with a control empty vector or a vector harboring the CaMKII-inhibitory protein (CaMKIIN $\alpha$ ), and relative luciferase activity determined as described above.

antibodies. Confirming the Western blots, we observed the highest level of both autonomous and total CaMK activity in the CaMKII $\gamma$  immunoprecipitates (Figure 1D, lane 5). Lower enzyme activity was detected in CaMKI immunoprecipitates (Figure 1D, lane 2), while minimal enzyme activity was observed in CaMKII $\alpha$ , CaMKII $\beta$ , CaMKII $\delta$ , and CaMKIV immunoprecipitates (Figure 1D).

*CaMKII $\gamma$  inhibits RAR activity in myeloid cells.* The studies described above indicate that CaMKII $\gamma$  is the CaMK predominantly expressed in myeloid cells, and we wished to determine whether this enzyme had any effect on RAR transcriptional activity. We used an expression vector harboring the full-length CaMKII as well as one harboring a truncated CaMKII that lacks the calmodulin-binding domain and displays constitutive activity (27). Since we observed that the CaMK inhibitor KN62 enhanced RAR transcriptional activity (Figure 1B),

we expected that the cotransfected CaMKII might inhibit RAR activity. Indeed this is the case, as we observed that both the full-length and the constitutively active CaMKII expression vectors significantly reduced RAR activity in the HL60 myeloid cells (Figure 2A, compare lanes 3–6 with lanes 1 and 2). Similar results were also noted in transfected MPRO and NB4 myeloid cells (not shown). We also determined the effect of siRNA-mediated downregulation of CaMKII $\gamma$  on RAR activity in HL60 cells. We constructed retroviral vectors expressing CaMKII $\gamma$  short hairpin RNAs (shRNAs), stably transduced HL60 cells with these vectors, and isolated 6 subclones (A6, B2, B5, B6, C2, and C18) that exhibited diminished protein expression of CaMKII $\gamma$  as compared with controls (Figure 2B). These subclones also exhibited diminished CaMKII $\gamma$  enzymatic activity as compared with control (empty) vector-transduced



**Figure 3** CaMKII directly interacts with RARα through an LxxLL binding motif. (A) HL60 cell lysates treated with ATRA (1 μM) for the indicated times were immunoprecipitated with the indicated antibodies followed by Western blot analysis. (B) In vitro-translated <sup>35</sup>S-labeled CaMKIIα was incubated with GST or a GST-RARα fusion protein attached to glutathione beads. The beads were washed and then subjected to SDS-PAGE. (C) Amino acid sequence indicating in boldface the conserved LxxLL motif in CaMKII. All 4 CaMKII isoforms (α, β, γ, and δ) harbor this identical sequence. In vitro-translated <sup>35</sup>S-labeled CaMKIIα cDNAs harboring the indicated mutations engineered within this LxxLL motif were subjected to GST pull-down assays using the GST-RARα fusion protein. (D) HL60 cells were electroporated with the βRARE-tk-Luc reporter (15 μg) together with expression vectors harboring parental CaMKIIα (WT) or CaMKIIα that harbors the indicated mutant LxxLL motif (LxxAA). After 6 hours of incubation with ATRA (1 μM), relative luciferase activity was determined on cell extracts.

HL60 cells (Figure 2C). The CaMKIIγ shRNA-transduced HL60 cells displaying this reduced CaMKIIγ expression/activity exhibited enhanced ATRA-induced RAR transcriptional activity as compared with the empty vector-transduced cells (Figure 2D). Moreover, the HL60 subclones exhibiting this reduced CaMKIIγ expression/activity displayed an enhanced differentiative response to RA, which was reflected in their enhanced RA-induced expression of Cd11b, a surface antigen present on mature neutrophils (28) (Figure 2E). Consistent with these observations, we observed that cotransfection of the RARE reporter with an expression vector harboring CaMKIINα, a naturally occurring protein that specifically inhibits CaMKII activity (29), enhanced RARE reporter activity in HL60 cells (Figure 2F). Taken together, these observations indicate that CaMKIIγ inhibits RAR transcriptional activity in myeloid cells.

*Physical interaction between CaMKIIγ and RARα is mediated through a CaMKII LxxLL motif.* Since the above observations indicate that CaMKIIγ regulates (inhibits) RAR transcriptional activity, we performed coimmunoprecipitation studies to determine whether there was any physical interaction between CaMKIIγ and RARα in myeloid cells. We observed that immunoprecipitation of endogenous RARα from both untreated and ATRA-treated HL60 cells coimmunoprecipitated CaMKIIγ (Figure 3A, lanes 3–5) and that antibodies to CaMKIIγ were able to coimmunoprecipitate RARα (Figure 3A, lanes 6–8). These coimmunoprecipitation studies indicate that there is a direct physical interaction between RARα and CaMKIIγ, and/or they are common members of a larger multiprotein complex. To determine whether CaMKII can directly bind RARα, we assessed the CaMKII/RARα interaction using in vitro glutathione S-transferase (GST) pull-downs. A GST-RARα fusion protein bound CaMKII, while GST alone did not (Figure 3B). Although a potential “bridge factor” in the reticulocyte lysate might encourage this RARα/CaMKII interaction, nevertheless these observations suggest that RARα and CaMKII can directly bind each other.

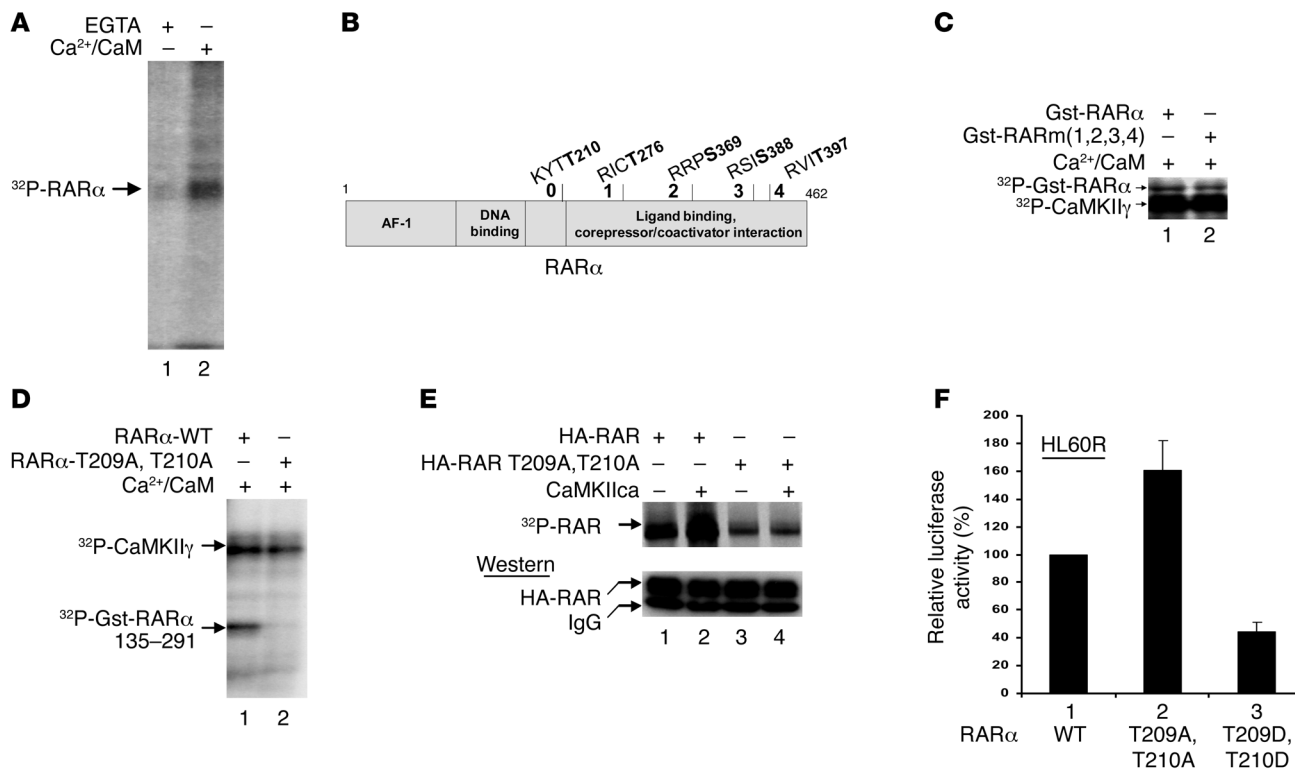
After ligand binding, a conformational change occurs in the RARs that promotes their interaction with a variety of different transcriptional co-regulators (coactivators). These coactivators harbor a sig-

nature LxxLL motif that directly mediates their interaction with RAR (7, 8). Inspection of the amino acid sequence of CaMKII indicates that the CaMKIIα, β, γ, and δ isoforms all harbor a single LxxLL motif (LyiLL) within the kinase domain (Figure 3C). To determine whether these LxxLL motif sequences are directly involved in mediating the interaction of CaMKII with RARα, we performed GST-RAR pull-downs using in vitro-translated CaMKII fusion proteins in which this LxxLL motif had been altered by site-directed mutagenesis. Mutation of LxxLL motif residues within CaMKII resulted in significantly reduced binding to GST-RARα (Figure 3C, compare lane 1 with lanes 2–6). Thus the CaMKII LxxLL motif appears to be important in mediating the binding of CaMKII to RARα.

To determine whether this LxxLL motif has any functional role in the CaMKII-mediated inhibition of RAR activity, we compared the activity of WT versus LxxLL motif-mutated CaMKII in regulating RAR reporter activity in transiently transfected HL60 myeloid cells. A CaMKII construct harboring a mutated LxxLL motif (LxxAA) that reduces binding to RAR (Figure 3C, lane 1 versus lane 3) did not inhibit RAR activity as compared with the parental (WT) CaMKII (Figure 3D). Thus the CaMKII LxxLL motif appears to be important in regulating both the physical and the functional interaction between CaMKII and RAR.

*CaMKIIγ directly phosphorylates RARα.* The direct binding of CaMKIIγ to RARα (Figure 3) together with the CaMKII-mediated regulation of RAR transcriptional activity (Figure 2) suggests that the RARs may be direct enzymatic substrates of the CaMKs. We performed in vitro kinase reactions and observed that CaMKII phosphorylated RARα in a Ca<sup>2+</sup>/CaM-dependent manner (Figure 4A). A consensus substrate sequence targeted by the different CaMKs is R-x-x-S/T (30), and RARα harbors 4 such candidate CaMK target sequences within the C-terminal ligand binding/coactivator interaction domain. (These RARα sites are arbitrarily labeled 1–4 in Figure 4B). To determine whether any of these 4 predicted substrate sites were phosphorylated by CaMKIIγ, we compared the CaMKII-mediated phosphorylation of GST-RARα fusion proteins in which all 4 of these potential phosphorylation sites





**Figure 4**

**Phosphorylation of RARα by CaMKIIγ.** (A) In vitro–translated RARα was subjected to an in vitro kinase reaction with in vitro–translated CaMKII. (B) The 4 consensus CaMK sites (R-x-x-S/T) within the ligand-binding domain of RARα are arbitrarily labeled 1–4. The (K-x-x-S/T) CaMK consensus site within the RARα hinge domain is labeled site 0. (C) A Gst-RARα fusion protein was constructed that harbors nonphosphorylatable alanine mutations at consensus CaMK sites 1–4 [Gst-RARm(1,2,3,4)]. This Gst-RARα fusion protein and the parental WT Gst-RARα were subjected to in vitro kinase reactions using CaMKIIγ immunoprecipitated from HL60 cells. The lower band corresponds to the immunoprecipitated CaMKIIγ that is autophosphorylated during this reaction. (D) A Gst-RARα fusion fragment harboring RARα amino acids 135–291 (RARα-WT) as well as the same fusion protein that is mutated (T→A) at RARα site 0 (RARα-T209A,T210A) was subjected to an in vitro kinase reaction using immunoprecipitated HL60 CaMKIIγ. The radiolabeled upper bands represent CaMKIIγ that is autophosphorylated during this reaction. (E) 293 cells were transfected with the indicated expression vectors and then metabolically labeled with [<sup>32</sup>P]orthophosphate. After 4 hours, anti-HA immunoprecipitates were electrophoresed on an agarose gel, and then radioautography was performed. CaMKIIca is the constitutively active CaMKIIα. Anti-HA Western blots (bottom row) served as a control for the amount of immunoprecipitated HA-RAR from each transfectant. (F) HL60R cells were electroporated with the βRARE-tk-LUC reporter (15 μg) together with expression vectors harboring the WT or the indicated CaMKII (T209,T210) site–mutated RARα. Six hours after RA addition (1 μM), relative luciferase activity was determined.

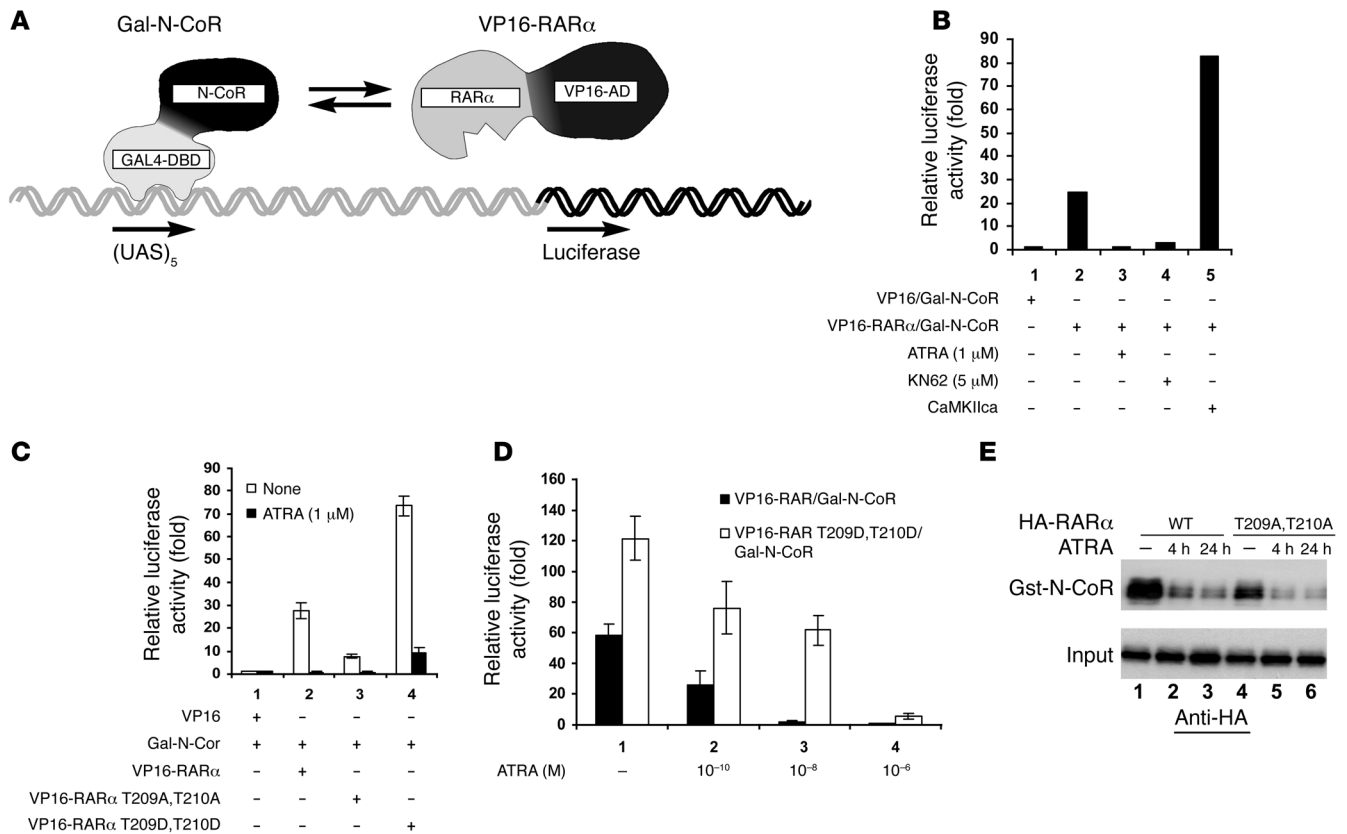
were mutated to alanine [designated Gst-RARm(1,2,3,4)]. None of these 4 consensus sites in RARα appeared to be phosphorylated by CaMKIIγ, since this enzyme phosphorylated both the WT and the Gst-RARα with 4 mutated consensus sites to an equal extent (Figure 4C, compare lanes 1 and 2). We identified another consensus CaMK site at T210 in the more N-terminal “hinge” region of RARα (arbitrarily designated site 0; see Figure 4B) that harbors a lysine rather than an arginine (KYTT210) (31). We found that immunoprecipitated CaMKIIγ readily phosphorylated a GST-RARα fusion protein harboring amino acids 135–291 of RARα but not the same fragment harboring a TT→AA mutation at this RARα site 0 (Figure 4D), indicating that the RARα site 0 is indeed a CaMKIIγ phosphorylation site.

To determine whether this CaMKIIγ phosphorylation site within RARα is also phosphorylated in vivo, we cotransfected <sup>32</sup>P-labeled 293 cells with constitutively activated CaMKII together with WT RARα versus the CaMKII site 0–mutated RARα. The transfected CaMKII enhanced the phosphorylation of the WT RARα (Figure 4E, compare lanes 1 and 2) but not the RARα that was mutated at the

T209,T210 site (Figure 4E, compare lanes 3 and 4). Thus the RARα CaMKII site that is phosphorylated in vitro (T209,T210) is also phosphorylated in vivo.

To determine whether CaMKIIγ-mediated phosphorylation of RARα at the T209,T210 site alters RARα activity, we compared the transcriptional activity of WT RARα versus RARα harboring mutations at this site. We performed these studies in HL60R cells, which harbor a mutated RARα resulting in reduced endogenous RAR activity (32). As compared with WT RARα, we observed that an RARα construct harboring nonphosphorylatable residues at the CaMKII site (T209A,T210A) displayed enhanced RA-induced activity, while the RARα harboring phosphorylation-mimicking mutations at this same site (T209D,T210D) exhibited reduced transcriptional activity (Figure 4F). Thus CaMKIIγ phosphorylation of RARα at the T209,T210 site inhibits RARα transcriptional activity.

In summary, we observe that CaMKIIγ directly phosphorylates RARα at a site within the hinge region of RARα (T209,T210), and this phosphorylation results in a downregulation of RARα transcriptional activity.



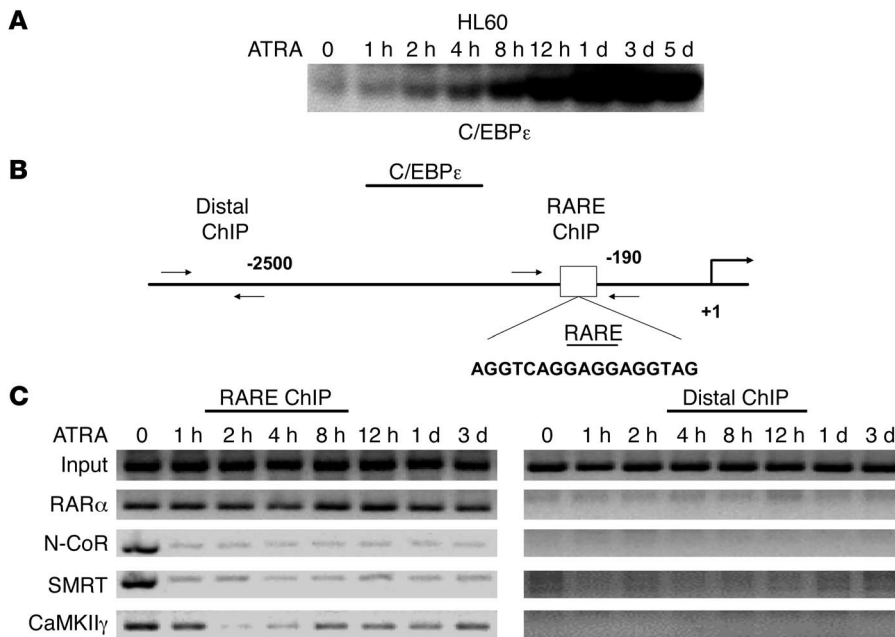
**Figure 5**

CaMKII phosphorylation of RARα enhances its interaction with transcriptional corepressors. (A) In the mammalian 2-hybrid approach, the activity of the transfected (UAS)<sub>5</sub> luciferase reporter is proportional to the interaction of Gal-N-CoR with VP16-RARα. (B–D) Relative luciferase activity of the transfected (UAS)<sub>5</sub> luciferase reporter was determined in lysates of cultured MPRO promyelocytes cotransfected with the indicated Gal-N-CoR and VP16-RARα WT versus VP16-RARα mutated expression vectors. Cells were then treated with the indicated concentration of ATRA for 6 hours. In B, KN62 treatment was for 48 hours. (E) Friend erythroleukemia cells that were stably transduced with the indicated HA-tagged WT RARα and CaMKII site–mutated RARα (T209A,T210A) were treated with ATRA (1 μM) as indicated, and pulldowns from these cell lysates using the Gst-N-CoR fusion protein were subjected to anti-HA Western blot analysis.

*CaMKIIγ phosphorylation of RARα enhances RARα interaction with transcriptional corepressors.* In the absence of RA, the RARs physically interact with the corepressors N-CoR and SMRT, and exposure to RA triggers corepressor release and coactivator recruitment leading to RAR transcriptional activation (8, 33). We used a mammalian 2-hybrid approach to determine whether the CaMKIIγ phosphorylation of RARα altered its interaction with corepressors and coactivators. Using constructs designed to assess the RARα/N-CoR interaction (Figure 5A), we observed that ATRA addition resulted in a marked decrease in the (UAS)<sub>5</sub>-driven luciferase reporter activity (Figure 5B, compare lanes 2 and 3), reflecting the previously observed ATRA-induced decrease in the RARα/N-CoR interaction (34). Importantly, treatment of the transfected cells with KN62 also significantly inhibited luciferase activity (Figure 5B, compare lanes 2 and 4), suggesting that the RARα/N-CoR interaction was also regulated by the CaMKs. Indeed, cotransfection of a CaMKII expression vector enhanced the luciferase reporter activity (Figure 5B, compare lanes 2 and 5), suggesting that CaMKII phosphorylation of RARα enhanced the RARα/N-CoR interaction. To directly test this hypothesis, we mutated the RARα CaMKII site (T209,T210) and observed that the T209A,T210A–mutated VP16-RARα fusion protein was associated with significantly reduced luciferase activity in the transfected cells as

compared with WT (Figure 5C, compare lanes 2 and 3). In contrast, a phosphorylation-mimicking VP16-RAR T209D,T210D mutant led to enhanced luciferase activity (Figure 5C, compare lanes 2 and 4). Moreover, compared with the WT VP16-RARα, this latter mutant required significantly higher concentrations of ATRA to diminish the RARα/N-CoR interaction (Figure 5D). Consistent with the mammalian 2-hybrid results, we noted that, with the use of lysates from cells stably transduced with RARα versus the CaMKII site–mutated RARα, a Gst-N-CoR fusion protein interacted with the WT RARα more efficiently than with the CaMKII site–mutated RARα (Figure 5E, compare lanes 1 and 4). Taken together, these observations indicate that CaMKIIγ phosphorylation of the RARα T209,T210 site enhances the RARα/N-CoR interaction.

*Association of CaMKIIγ with RA-responsive myeloid gene promoters.* The above observations indicate a potentially important role for CaMKIIγ in directly phosphorylating RARα and regulating its transcriptional activity in myeloid cells. We used chromatin immunoprecipitation (ChIP) to determine whether CaMKIIγ directly associated with the promoters of RAR-regulated target genes. For these studies we chose to analyze the CCAAT/enhancer binding protein-ε (C/EBPε) gene in HL60 cells, since enhanced C/EBPε expression occurs during RA-induced differentiation of



**Figure 6**

RA induces changes in the association of CaMKII $\gamma$  with the C/EBP $\epsilon$  promoter RARE. (A) Western blot of cell lysates exhibiting enhanced C/EBP $\epsilon$  expression in ATRA-treated (1  $\mu$ M) HL60 cells. (B) The human C/EBP $\epsilon$  5' region indicating the location of the ChIP PCR primers. (C) ChIP assays. HL60 cell chromatin from cells treated with ATRA (1  $\mu$ M) for the indicated times was immunoprecipitated with the indicated antibodies, and the DNA was isolated and subjected to PCR using the indicated C/EBP $\epsilon$  promoter primer pairs.

HL60 (Figure 6A), and this is mediated through a specific RARE located 190 bp upstream of the C/EBP $\epsilon$  translation start site (35). For these ChIPs, PCR primers flanking the C/EBP $\epsilon$  RARE were used together with control primers flanking a distal C/EBP $\epsilon$  genomic region approximately 2,500 bp upstream (Figure 6B). Both before and after RA induction we observed a consistent association of RAR $\alpha$  with the C/EBP $\epsilon$  RARE but not with the upstream distal region (Figure 6C). Following RA treatment there was the expected rapid displacement from this RARE of the RAR corepressors N-CoR and SMRT (Figure 6C). We observed that CaMKII $\gamma$  was also associated with this RARE in uninduced cells as compared with the upstream control region. Interestingly, RA treatment was accompanied by dissociation of CaMKII $\gamma$  from this RARE, and these changes were observed as early as 1–2 hours after RA exposure (Figure 6C).

The CaMK inhibitor KN62 is a potent inducer of myeloid cell differentiation. The RA-induced myeloid differentiation of HL60 cells is mediated directly through RAR $\alpha$  (36). Our observation that KN62 enhanced RAR transcriptional activity in HL60 cells (Figure 1B) suggests that this compound might also regulate the differentiation of these cells. We observed that KN62 as a single agent was a potent inducer of HL60 differentiation, enhancing both morphologic differentiation and expression of the neutrophil antigen Cd11b (Figure 7, B and D). Similarly, as a single agent, KN62 enhanced the granulocytic differentiation of the MPRO cell line (not shown). In contrast with the HL60 and MPRO cells, we observed in the NB4 promyelocytic leukemia cell line, which harbors the PML-RAR $\alpha$  fusion gene (22), that KN62 alone was a relatively poor inducer of morphologic granulocytic differentiation (Figure 7, compare panels E and G). However, KN62 in combination with relatively low concentrations of RA (10<sup>-9</sup> to 10<sup>-10</sup> M)

induced a marked increase in the granulocytic differentiation of NB4 cells, as assessed by morphologic differentiation (Figure 7, compare panels G and H), as well as the induction of Cd11b surface antigen expression (Figure 7I). Thus the CaMK inhibitor KN62 not only enhances RAR transcriptional activity but, either as a single agent or in combination with relatively low doses of RA, can induce or enhance the differentiation of different myeloid leukemia cell lines.

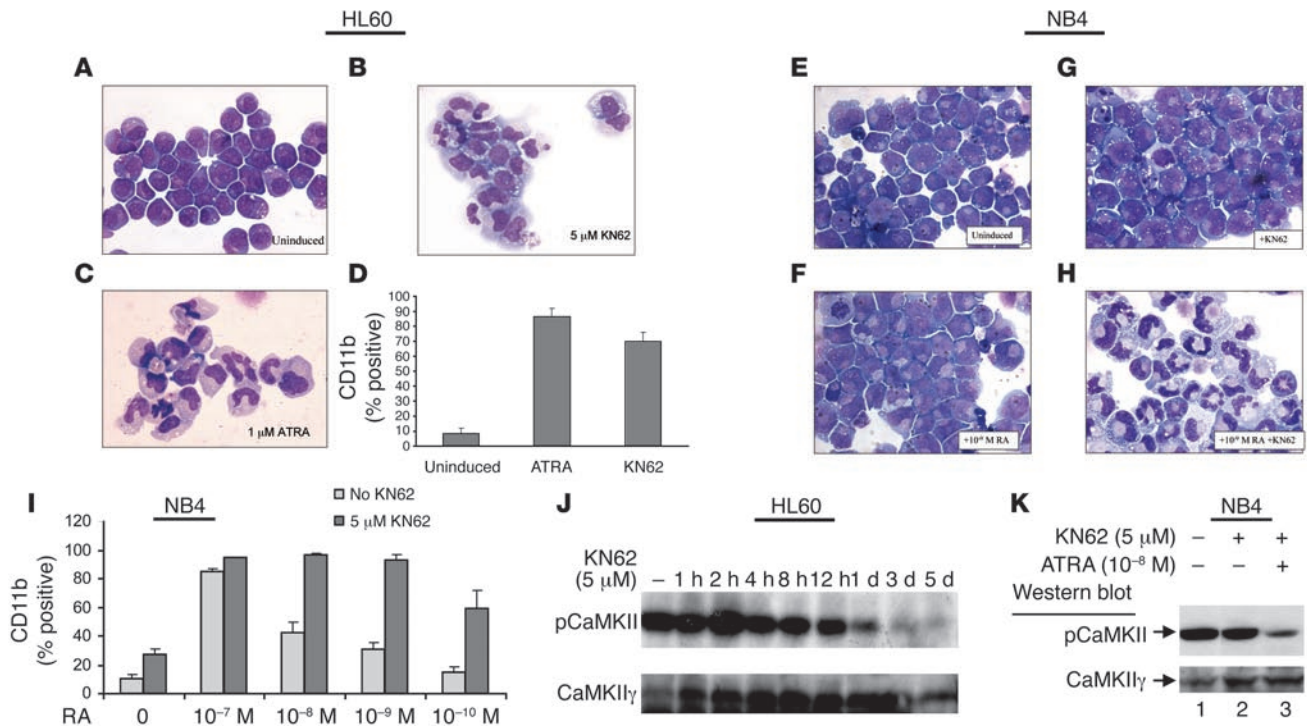
KN62 can inhibit multiple different CaMKs by interfering with CaMK binding to Ca<sup>2+</sup>/CaM. To determine whether KN62 might alter CaMKII activation in differentiating myeloid cells, we used phosphospecific CaMKII antibodies that identify the activated CaMKII. Autophosphorylation of threonine 286/287 (CaMKII $\alpha$ : T286; CaMKII $\beta$ ,  $\gamma$ , and  $\delta$ : T287) of CaMKII is associated with autonomous enzyme activation (37), and this autophosphorylated CaMKII can be detected with the use of a CaMKII T286/287 phosphospecific antibody. In HL60 cells, KN62 induced a marked reduction in the activated, autophosphorylated CaMKII $\gamma$  that was readily apparent within a day following KN62 exposure (Figure 7J). In contrast,

KN62 did not induce any significant changes in the expression of total CaMKII $\gamma$  in these differentiating cells (Figure 7J). In NB4 cells, KN62 alone had little effect on the levels of autophosphorylated CaMKII $\gamma$ , but the combination of KN62 and relatively low concentrations of RA, which induced NB4 differentiation (Figure 7H), also induced a reduction in the autophosphorylated CaMKII $\gamma$  (Figure 7K). Thus the KN62-mediated enhanced differentiation of HL60 and NB4 cells is associated with reduced amounts of the activated, autophosphorylated CaMKII $\gamma$ .

## Discussion

CaMKII $\gamma$  is a member of the RAR transcription complex. Our studies have revealed a novel, previously unexplored cross-talk between Ca<sup>2+</sup>-regulated and RAR signal transduction pathways. Indeed, we observe that CaMKII, which is one of the most widely studied Ca<sup>2+</sup>-regulated enzymes and which is a critical regulator of neuronal cell development and activity, is an important regulator of RAR transcriptional activity in myeloid cells. Indeed, a number of our observations indicate that CaMKII $\gamma$  is a direct functional component of the RAR transcription complex. First, we observe in transient transfection assays that CaMKII inhibits the transcriptional activity of the RARs (Figure 2A). Moreover, CaMKII directly binds to the RARs through a CaMKII signature LxxLL motif (Figure 3C), a motif also present in nuclear receptor transcriptional coactivators (8). Finally, our ChIP studies indicate that CaMKII $\gamma$  localizes in vivo to RAREs within myeloid target gene promoters (Figure 6). Notably, RA treatment triggers a reduced association of CaMKII $\gamma$ , which inhibits RAR activity, with this RARE (Figure 6C). This RA-induced reduction in CaMKII $\gamma$  association with the RARE



**Figure 7**

KN62 regulates the differentiation of myeloid leukemia cell lines. (A–D) Wright-Giemsa–stained HL60 cells either uninduced (A) or treated for 5 days with KN62 (B) or ATRA (C). (D) CD11b expression in HL60 cells after 5 days of exposure to KN62 (5  $\mu$ M) or ATRA (1  $\mu$ M). (E–H) Wright-Giemsa–stained NB4 cells treated for 5 days with KN62 (5  $\mu$ M) and ATRA as indicated. Uninduced NB4 cells are primarily immature myeloblasts (E), and their differentiation is not significantly altered by KN62 treatment alone (G). NB4 cells treated with low-concentration ATRA (10<sup>-9</sup> M) display little differentiation (F), but granulocytic differentiation is markedly enhanced with the addition of KN62 (5  $\mu$ M) (H). Original magnification,  $\times$ 500. (I) CD11b expression of NB4 cells treated for 5 days with the indicated concentrations of RA and/or KN62. (J) Western blotting with the CaMKII $\gamma$  antibody, as well as the phosphospecific CaMKII (T286/287) antibody (pCaMKII), was performed on HL60 cell lysates after incubation with KN62 for the indicated times. (K) Western blots of NB4 cell lysates after a 5-day incubation with the indicated compounds.

may serve to relieve the inhibitory effect of CaMKII $\gamma$  on RAR activity and may be an important mechanism to regulate or amplify RAR activity following RA induction. Interestingly, some CaMKII $\gamma$  appears to return to the promoter 4–8 hours after ligand addition (Figure 6C), which may be indicative of the cyclical interaction of corepressors and coactivators previously observed at the gene targets of other nuclear hormone receptors (38).

*CaMKII $\gamma$  phosphorylation of RAR $\alpha$  enhances its interaction with transcriptional corepressors.* The multicomponent RAR transcription complex may consist of at least 30–40 proteins, and multiple substrates may be involved in the CaMKII-mediated downregulation of RAR activity. Nevertheless, our experimental observations indicate that the RARs themselves appear to be a critical substrate of CaMKII $\gamma$ . Indeed, we have identified a target site within RAR $\alpha$  (T209, T210) that is directly phosphorylated by CaMKII $\gamma$  both in vitro and in vivo. This site is evolutionarily conserved in all of the different RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$  isoforms from multiple different species and lies within the “linker” region of RAR that separates the DNA-binding and AF-2 domains of RAR. We observe that CaMKII $\gamma$ -mediated phosphorylation at RAR $\alpha$  T209, T210 enhances the RAR $\alpha$  interaction with the N-CoR transcriptional corepressor (Figure 5), which likely leads to reduced RAR transcriptional activity. This CaMKII site likely does not make direct contact with N-CoR, since corepressor interaction with RAR $\alpha$  appears to map more C-terminal to this site (39, 40). Rather, phosphorylation of this CaMKII site within the

RAR $\alpha$  linker region likely results in a steric change in RAR $\alpha$  that enhances its interaction with N-CoR. The direct binding of N-CoR to the RAR is a critical mediator of RAR transcriptional repression, since N-CoR acts as a scaffold protein to recruit different transcriptional repressors including the histone deacetylases to RA-responsive promoters (8). Thus this CaMKII $\gamma$ -mediated enhancement of the RAR $\alpha$ /N-CoR interaction likely explains the CaMKII $\gamma$ -mediated inhibition of RAR $\alpha$  transcriptional activity.

*Pharmacological CaMK inhibitors trigger myeloid leukemia cell differentiation.* Particularly compelling evidence for the physiological importance of the CaMKII-mediated regulation of RAR activity is our experimental observation that KN62, a pharmacological inhibitor of the CaMKs, enhances both RAR transcriptional activity (Figure 1B) and the differentiation of certain myeloid leukemia cell lines (Figure 7). Acute myelogenous leukemia is a heterogeneous disease, and successful therapy with RA has been generally confined to those leukemias harboring RAR $\alpha$  chromosome translocations. Indeed, our preliminary studies indicate that KN62 does not induce the differentiation of myeloid leukemia cell lines such as K-562, KCL-22, and Kasumi that do not harbor such RAR $\alpha$  chromosome translocations and are generally unresponsive to RA (not shown). Moreover, in contrast with WT HL60, we do not observe any KN62-mediated differentiation of HL60R cells, which harbor a mutated RAR $\alpha$ , or of NB4 subclones that are unresponsive to ATRA. These initial observations suggest





that any therapeutic application of KN62 may be limited to those myeloid leukemias that display at least some sensitivity to the differentiative effects of RA.

How does KN62 trigger the differentiation of certain myeloid leukemia cells? Since KN62 inhibits the binding of  $\text{Ca}^{2+}/\text{CaM}$  to the CaMKs, this compound can potentially inhibit the multiple different CaMKs, which are all activated by  $\text{Ca}^{2+}/\text{CaM}$  (41). However, our data strongly suggest that CaMKII $\gamma$  is a critical KN62 target for HL60 differentiation, since CaMKII $\gamma$  is the predominant CaMK expressed in myeloid cell lines (Figure 1, C and D), CaMKII inhibits RAR transcriptional activity (Figure 2A), and RNA interference-mediated inhibition of CaMKII $\gamma$  enhances both RA-induced RAR activity and RA-induced myeloid differentiation (Figure 2, D and E). Moreover, in HL60 cells, which harbor a constitutively activated (autophosphorylated) CaMKII $\gamma$ , KN62 inhibits this CaMKII $\gamma$  autophosphorylation (Figure 7J). Indeed, our studies would suggest that pharmacological or peptide inhibitors that are selective for CaMKII $\gamma$  may be of particular value in the therapy of certain myeloid leukemia cells.

## Methods

**Antibodies and chemical reagents.** Antibodies against RAR $\alpha$ ,  $\beta$ -tubulin, CaMKII $\alpha$ , CaMKII $\beta$ , CaMKII $\gamma$ , CaMKII $\delta$ , and CaMKI $\alpha$  as well as phosphospecific antibodies against CaMKII were all purchased from Santa Cruz Biotechnology Inc. Antibodies against N-CoR and SMRT were from Upstate Biotechnology. CaMKIV antibody was from BD Biosciences. Anti-FLAG antibody was from Sigma-Aldrich and anti-HA antibody from Novabiochem. Quantitation of CD11b surface antigen expression was performed with a fluorescence-activated cell sorter (FACS) with PE-conjugated anti-CD11b antibody from BD Biosciences. KN62, KN93, and ionomycin were obtained from Calbiochem. ATRA and calmodulin agarose beads were from Sigma-Aldrich.

**Protein extracts, Western blotting, and coimmunoprecipitations.** Western blotting and coimmunoprecipitations were performed as previously detailed (42).

**CaMK assays.** To assess the activity of immunoprecipitated CaMKs, the immunoprecipitates were incubated for 8 minutes at 30°C in 40  $\mu\text{l}$  of a buffer containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 20 mM  $\beta$ -glycerol phosphate, 0.5 mM DTT, 50  $\mu\text{M}$  cold ATP, 1  $\mu\text{M}$  protein kinase A inhibitor (Sigma-Aldrich), 1  $\mu\text{M}$  protein kinase C inhibitor (Sigma-Aldrich), and 10  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP (3,000 Ci/mmol) (PerkinElmer Life and Analytical Sciences). For assessment of  $\text{Ca}^{2+}/\text{CaM}$ -independent activity the above buffer contained 1 mM EGTA. For assessment of  $\text{Ca}^{2+}/\text{CaM}$ -dependent activity the buffer contained 1 mM  $\text{CaCl}_2$ , 1  $\mu\text{M}$  calmodulin (Calbiochem) without EGTA. The peptide substrate included autocalmitide-2 (KKALRRQET\*VDAL) (43) to assess CaMKII activity, syntide-2 (PLARTLS\*VAGLPGKK) (44) to assess CaMKI, and the aa 345–358 fragment of CaMKII $\delta$  (KSDGGVKKRKS\*SS) to assess CaMKIV activity. After incubation the phosphorylated substrate was separated from the residual [ $\gamma$ - $^{32}\text{P}$ ]ATP with the use of P81 phosphocellulose paper and quantitated with a scintillation counter. For the in vitro kinase assays, either in vitro-translated or immunoprecipitated CaMKs were incubated with bacterially purified GST-RAR $\alpha$  fusion proteins attached to glutathione beads. The beads were washed, boiled, and then subjected to SDS-PAGE.

**ChIP.** HL60 cells incubated for various periods of time after ATRA treatment (1  $\mu\text{M}$ ) were harvested and cross-linked with the use of 1% formaldehyde for 15 minutes at 37°C and incubated another 15 minutes at room temperature with 125 mM glycine. Cells then were rinsed and washed 3 times with cold PBS. The cell pellets were resuspended in 0.2–2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitors (1  $\mu\text{g}/\text{ml}$  leupeptin, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1 mM PMSF, 1  $\mu\text{g}/\text{ml}$  pep-

statin A, 10  $\mu\text{M}$  antipain, 10  $\mu\text{M}$  bestatin, 1 mM NaF, and 1 $\times$  phosphatase inhibitor cocktail I/II) and sonicated 3 times at 20 seconds each to reduce DNA length to 200–800 bp. Supernatants were collected by centrifugation for 10 minutes at 10,000  $g$  at 4°C and diluted in 10-fold dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) with the above protease inhibitors. The diluted cell extracts (500–1,000  $\mu\text{g}$ ) were incubated with 4  $\mu\text{g}$  or 10  $\mu\text{l}$  antibodies overnight at 4°C followed by 50  $\mu\text{l}$  of salmon sperm DNA/protein A/G-Sepharose beads for 4 hours. Pellet beads were washed sequentially for 5 minutes each in buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), buffer B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), buffer C (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer. Immune complexes were eluted with 1% SDS, 0.1 M  $\text{NaHCO}_3$ , and incubated at 65°C overnight to reverse the cross-linking. DNA fragments were isolated with a QIAquick spin column (QIAGEN). PCR was performed for 20–30 cycles. PCR primers flanking the C/EBP $\epsilon$  promoter RARE used for the “RARE ChIP” include 5'-AAGAGAGGCATTGACTAC-3' (forward) and 5'-ACCTGCTCTTGAGGCACCCCTT-3' (reverse). Primers used for the upstream control region “distal ChIP” include 5'-CCTGACATCCAGAGCCCTGCCT-3' (forward) and 5'-AGTAGCTTCAGTGAAGTGGGCAC-3' (reverse).

**CaMK RNA interference constructs.** Separate cassettes harboring the U6 (–332 to +1) or H1 (–345 to +1) promoters were inserted into the EcoRI-BaMHI cloning site of the LXSN retroviral vector (M28248). These cassettes harbor sites into which synthetic oligonucleotides corresponding to sense and antisense strands of human CaMKII $\gamma$  separated by a 9-bp spacer harboring an XhoI site (5'-TTCTCGAGA-3') were cloned. Three different sequences from the human CaMKII $\gamma$  coding region (NM\_172172) were chosen (453–473, 618–639, and 790–812) and arbitrarily labeled A, B, and C. These sequences were cloned into the above vectors, which were then transfected into the PT67 packaging cell line, and the retroviral particles were used to infect HL60 cells as previously detailed (36). G418-resistant HL60 clones were isolated and screened by Western blotting to compare CaMKII $\gamma$  expression levels. Those HL60 subclones exhibiting reduced CaMKII $\gamma$  expression (i.e., A6, B2, B5, B6, C2, and C18) were further analyzed for immunoprecipitable CaMKII activity, RAR transcriptional activity, and differentiative response to RA.

**Transient transfections and luciferase assays.** Murine PT67 fibroblasts were transfected with the use of calcium phosphate precipitation. All hematopoietic cell lines were transiently transfected by electroporation as previously detailed (45). In the transient transfections the LXSN vector (M28248) served as the expression vector for the WT and site-directed mutagenized CaMKII and RAR $\alpha$  coding region cDNAs. Human cDNA homologous to the coding region of rat CaMKIIN $\alpha$ , a CaMKII-inhibitory protein (29), was obtained by RT-PCR from HL60 RNA and cloned into the LXSN vector. The RA-responsive luciferase reporter harbors the RARE from the RAR $\beta$ 2 promoter, as previously detailed (42). The PON838 plasmid, a  $\beta$ -gal reporter driven by the  $\beta$ -actin promoter, was used as an internal control to determine transfection efficiency for calculation of relative luciferase activity.

**Mammalian 2-hybrid constructs.** The luciferase reporter harboring 5 Gal4-binding sites was previously described (45). The complete coding sequence of the human RAR $\alpha$  (NM\_000964) was amplified by PCR and cloned into the PSG-VP16 vector (46). The N-CoR C-terminal receptor-interacting domain (amino acids 1,946–2,453) was amplified by PCR and cloned into the EcoRI site of the GAL<sub>dbd</sub>(1–147) expression vector, pSG424 (47).

**Site-directed mutagenesis.** Site-directed mutagenesis on the different CaMK and RAR $\alpha$  or VP16-RAR $\alpha$  constructs was performed with the QuikChange procedure (Stratagene).

**GST pulldowns.** GST fusion proteins were purified from bacteria with the use of glutathione-4B Sepharose (Amersham Biosciences).



<sup>35</sup>S-labeled proteins were translated in vitro with a T<sub>7</sub> Quick Coupled transcription/translation system (Promega). The products were incubated overnight at 4°C with the GST fusion proteins attached to the glutathione Sepharose beads. The beads were then washed, boiled, and subjected to SDS-PAGE.

### Acknowledgments

This work was supported by NIH HL54881, NIH RO1 CA118971, and the Leukemia and Lymphoma Society (grant 6144). We thank Anthony Means for providing CaMK cDNA vectors and Sara

Hook, Bob Eisenman, and Jon Cooper for reading this manuscript and providing helpful comments.

Received for publication October 27, 2006, and accepted in revised form February 13, 2007.

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