Ca^2+/Calmodulin-dependent Protein Kinase IV Stimulates Nuclear Factor-κB Transactivation via Phosphorylation of the p65 Subunit*

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CaM-dependent protein kinase IV (CaMKIV) is a key mediator of Ca^{2+}-induced gene expression. In this study, CaMKIV was found to directly associate with and phosphorylate the nuclear factor-κB (NFκB) component p65 both in vitro and in vivo. The phosphorylation of p65 by CaMKIV resulted in recruitment of transcription co-activator cAMP-response element-binding protein-binding protein and concomitant release of corepressor silencing mediator for retinoid and thyroid hormone receptors, as demonstrated by the glutathione S-transferase pull down and mammalian two hybrid assays. In addition, cotransfection of CaMKIV resulted in cytosolic translocation of the silencing mediator for retinoid and thyroid hormone receptors. Consistent with these results, cotransfected CaMKIV dramatically stimulated the NFκB transactivation in mammalian cells. From these results, NFκB is suggested to be a novel downstream effector molecule of CaMKIV.

Calmodulin (CaM) is the most ubiquitous and abundant Ca^{2+}-binding protein in cells that is an essential protein that serves as a receptor to sense changes in calcium concentrations and, in this fashion, mediates the second messenger role of this ion (reviewed in Ref. 1). Calcium binds to CaM by means of a structural motif called an EF-hand, and a pair of these structures is located in both globular ends of the protein. CaM binds to and activates target enzymes. These Ca^{2+}/CaM-dependent protein kinases include CaM-kinase kinase, CaMKI and CaMKII. CaMKIV is a monomeric multifunctional enzyme that is expressed only in subnucleosomal portions of the brain, T lymphocytes, and postmeiotic male germ cells. CaMKIV is present in the nucleus of the cells in which it is expressed and has been implicated in regulation of transcription of a number of genes including those encoding interleukin 2, members of the immediate early gene family such as c-fos, tumor necrosis factor family members such as CD40L, FasL, and tumor necrosis factor, the neurotrophin, BDNF, an Epstein-Barr virus gene involved in the switch to the lytic cycle called BZLF1, and orphan members of the steroid receptor superfamily such as ROR and COUP-TF (1). However, the only direct substrates for CaMKIV involved in transcription that have been defined to date are CREB and CREM (2, 3) although the transcription coactivator CREB-binding protein (CBP) has also been indirectly implicated as a possible substrate (4).

Nuclear factor-κB (NFκB), composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family of polypeptides, is important for the inducible expression of a wide variety of cellular and viral genes (reviewed in Ref. 5). In vertebrates, this family comprises p50, p65 (RelA), c-rel, p52, and RelB. These proteins share a 300-amino acid region known as the Rel homology domain, which binds to DNA and mediates homo- and heterodimerization. This domain is also the target of the IκB proteins, which include 1κBα, 1κBβ, 1κBγ, Bcl-3, p105, and p100. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins (5). In response to various inducers, a multisubunit protein kinase, the IκB kinase, is rapidly activated and phosphorylates two critical serine residues in the N-terminal regulatory domain of the IκBs. Phosphorylated IκBs are recognized by a specific E3 ubiquitin ligase complex and undergo polyubiquitination, which targets them for rapid degradation by the 26 S proteasome. NFκB dimers, which are spared from degradation, translocate to the nucleus to activate gene transcription (5).

Transcriptional coactivators either bridge transcription factors and the components of the basal transcriptional apparatus and/or remodel the chromatin structures (reviewed in Ref. 6). In particular, CBP and its functional homologue p300, as well as steroid receptor coactivator-1 and its family members were shown to be essential for the activation of transcription by a large number of regulated transcription factors, including NFκB (7–9). Interestingly, steroid receptor coactivator-1 and its homologue ACTR, along with CBP and p300, were recently shown to contain histone acetyltransferase activities and associate with yet another histone acetyltransferase protein P/CAB (6). In contrast, nuclear receptor corepressor (N-CoR) and its homologue-silencing mediator for retinoid and thyroid hormone receptors (SMRT) harbor transferable repression domains that can associate with various histone deacetylases (HDAC). In humans, three highly homologous class I (HDAC1, HDAC2, and HDAC3) and four class II (HDAC4, HDAC5, HDAC6, and HDAC7) HDAC enzymes have been identified to date. The class I deacetylases HDAC1 and HDAC2 are compo-
nents of multisubunit complexes mSin3A and the NuRD complex (10, 11). It is interesting to note that N-CoR/SMRT serves as an adapter molecule between the core mSin3 complex and sequence-specific transcriptional repressors without stably associating with the mSin3 complex. More recently, however, SMRTN-CoR was found to be a direct component of a newly isolated HDAC3 complex (12, 13). N-CoR and SMRT have also been reported to partner with HDAC4, HDAC5, and HDAC7 (14, 15). These results are consistent with the notion that acetylation of histones destabilizes nucleosomes and relieves transcriptional repression by allowing transcription factors to access to recognition elements, whereas deacetylation of the histones stabilizes the repressed state (6). Interestingly, N-CoR/SMRT is also known to mediate transcriptional repression from a wide variety of other non-receptor-mediated pathways (16–21). These include AP-1, NFκB, SRF, MyoD, the bHLH-LZ proteins Mad and Mxi that mediate repression of Myc activities and tumor suppression, E2F-repressive retinoblastoma protein, and the oncoproteins PLZF-RAR and LAZ3/BCL6, which are involved in acute promyelocytic leukemia and non-Hodgkin’s lymphomas, respectively.

In this work, we found that CaMKIV specifically interacted with and phosphorylated the NFκB component p65, and the CaMKIV-mediated phosphorylation of p65 resulted in enhanced recruitment of CBP with concomitant dissociation of SMRT. In addition, cotransfection of CaMKIV resulted in cytosolic translocation of SMRT. Accordingly, CaMKIV dramatically stimulated the NFκB transactivation. From these results, we concluded that NFκB is a novel downstream effector molecule of CaMKIV.

EXPERIMENTAL PROCEDURES

Plasmids, Chemicals, Cells, and Antibodies—The polymerase chain reaction-amplified fragments for the full-length CaMKIV, CaMKIVc (the CaMKIV residues 1–313), and CREB were subcloned into EcoRI and XhoI (or Sall) restriction sites of the LexA fusion vector pEG202PL, the B42 fusion vector pG4–5, the mammalian two hybrid vectors pCMX/Gal4 and pCMXVP16, and the mammalian expression/in vitro translation vector pcdNA3. The polymerase chain reaction-amplified fragments for CaMKIV, CaMKIVc, human p65, and p65C (the p65 residues 431–551) were inserted into EcoRI and XhoI restriction sites of the glutathione S-transferase (GST) fusion vector pGEX4T-1. Expression vectors for various CaMKs and their C-terminal deleted forms with constitutive activities were obtained from Dr. Tony Means at Duke University. The mammalian two hybrid vectors pCMXVP16-SMRT-D, pCMXVP16-CPB-A, pCMX/Gal4-p65, and pCMXVP16-p65, the mammalian expression vectors for p65, CBP, CBP-A, SMRT-D, GFP,SMRT, and a constitutively active form of MEKK-1, and the yeast expression vectors encoding B42 fusions to p50 and p65C were as described (9, 16, 22–24). CV-1 and HeLa cells were obtained from ATCC (Manassas, VA), and a constitutively active form of MEKK-1, and the yeast expression vectors pCMX/VP16-CBP-A, pCMX/Gal4-p65, and pCMX/VP16-p65, the mammalian expression/vectors for various CaMKs and their C-terminal deleted forms with constitutive activities were obtained from Dr. Tony Means at Duke University.

Cell Culture, Transfection, and Microscopy—HeLa and CV-1 cells (5 × 10⁵ cells/well) were grown in 24-well plates with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h and transiently transfected using SuperFect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The cells were harvested 48 h later, the luciferase assays were done as described (25), and the results were normalized to the LacZ expression. For treatment with ionomycin and KN-93, the cells, 24 h post-transfection, were replaced with fresh medium containing ionomycin or KN-93. The cells were lysed 24 h later. For the localization studies of SMRT, ~10⁶ CV-1 cells were seeded in a chambered coverslip cell culture system (Nalge-Nunc, Rochester, NY) and were transfected with the pCMV-GFP-SMRT vector (24), together with an appropriate expression vector for the activated form of MEKK-1 (24) or various CaMKs, as indicated (or an equivalent empty vector as a control) using the SuperFect procedure. One day after transfection, the subcellular location of the GFP-SMRT fusion polyopeptide was visualized using a Zeiss Axioskop 2 microscope.

The Yeast β-Galactosidase Assay—The cotransformation and β-galactosidase assay in yeast were done as described (25). For each experiment, at least three independently derived colonies expressing chimeric proteins were tested.

GST Pull Down Assays—Equal amounts of GST alone or GST fusion proteins, expressed in Escherichia coli and purified, were bound to glutathione and incuose 4B and incubated in icecold reduced buffer (5 mM NaCl, 25 mM Hepes (pH 7.9), 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, and 1.5% bovine serum albumin) with labeled proteins expressed by in vitro translation by using the TNT-coupled transcription-translation system, with conditions as described by the manufacturer (Promega, Madison, WI). Specifically bound proteins were eluted from beads with 40 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) and analyzed by SDS-PAGE, and autoradiography as described (25). Phosphorylation in Vivo and In Vitro—For in vivo phosphorylation, HeLa cells were cultured in 100-mm dish at density of 1 × 10⁶ cells/ml for 24 h, transfected with 5 μg of the indicated expression vectors using SuperFect, grown for 24 h, starved in serum-free Dulbecco’s modified Eagle’s medium for 30 min, and treated with 100 μM of ATP or [γ-32P]ATP for labeling. CaMKIV was expressed by in vitro translation by using the TNT-coupled transcription-translation system and purified by immunoprecipitation with CaMKIV-specific antibody. Alternatively, HeLa cells cotransfected with indicated expression vectors were lysed, immunoprecipitated with p65 antibody, washed three times with phosphate-buffered saline, and utilized as a source for putative p65-associated kinase(s) in vivo. Kinase reactions were carried out for 30 min at 30 °C with 25 mM Hepes (pH 7.6), 10 mM MgCl₂, 5 μg [γ-32P]ATP, 1 mM dithiothreitol, and 5% glycerol. To activate the full-length CaMKIV, 1 mM CaCl₂ and 10 μg/ml of CaM were added.

RESULTS AND DISCUSSION

The Direct Interaction of CaMKIV with NFκB—It is interesting to note that there are significant overlaps in function between NFκB and CaMKIV, such as their involvement in apoptosis and pro-proliferation (1, 5). Furthermore, CaMKIV is known to reside in the nucleus in vivo and directly phosphorylate the transcription factors CREB and CREM (2, 3). Thus, we tested whether CaMKIV is also functionally linked to NFκB. As shown in Fig. 1A, the constitutively active mutant form of CaMKIV consisting of the CaMKIV residues 1–313 (i.e. CaMKIVc) was found to interact with the NFκB components p50 and p65 in yeast. In the case of p65, p65C consisting of the C-terminal transactivation domain of p65 (i.e. the p65 residues 431–551) was utilized, because the full-length p65 was not readily expressed. As expected, the positive control CREB was also found to directly interact with CaMKIVc in yeast. Similar results were also obtained with the full-length CaMKIV (data not shown). Consistent with these results, coexpression of a fusion protein consisting of the transactivation domain VP16 and CaMKIVc stimulated the Gal4/p65-directed transactivation in HeLa cells, suggesting that p65 directly interacts with CaMKIV in vivo (Fig. 1B). In this experiment, it is notable that 20 μM of the CaMK inhibitor KN-93 was used, because VP16/ CaMKIVc alone was an effective stimulator of the Gal4/p65 transactivation in the absence of KN-93 (data not shown). Coexpression of VP16/p65 or p65 alone stimulated the Gal4/p65-mediated level of transactivation directed by the Gal4-Luc reporter construct (data not shown). Furthermore, labeled p65 protein expressed by using the TNT-coupled in vitro transcription-translation system specifically interacted with GST fusions to the full-length CaMKIV and CaMKIVc but not with GST alone (Fig. 1C). Interestingly, the p50-CaMKIV interaction was much weaker in the mammalian two hybrid tests and not readily observed in the GST pull down assays (data not shown), sug-
Fig. 1. Direct interactions of CaMKIV and NFκB. A, the indicated B42 and LexA plasmids were transformed into a yeast strain containing an appropriate lacZ reporter gene as described (25). Open and closed boxes indicate the presence of LexA alone and LexA fusion to CaMKIVc, respectively. The p65 residues 431–551 were included in p65C. The data are representative of at least two similar experiments, and the error bars are as indicated. B, HeLa cells were transfected with LacZ expression vector and VP16 fusion to CaMKIVc, along with an expression vector for Gal4/p65 and a reporter gene, Gal4-Luc, as indicated. Normalized luciferase expressions from triplicate samples were calculated relative to the input. C, the full-length p65 was labeled with [35S]methionine by in vitro translation and incubated with glutathione beads containing GST alone, GST/CaMKIV, and GST/CaMKIVc, as indicated. Beads were washed, and specifically bound material was eluted with reduced glutathione and resolved by SDS-PAGE. Approximately 20% of the total reaction mixture was loaded as input.

Consistent with the lack of its direct interactions with CaMKIV in vitro, whereas the overexpression of the protein revealed some weak labeling with the full-length p65 (data not shown). These results suggest that efficient phosphorylation of the full-length p65 in vivo may require other additional proteins or signaling events that are not present in the in vitro reactions. Nevertheless, these results clearly demonstrate that the C-terminal transactivation domain of p65 is a direct phosphorylation substrate of CaMKIV. Finally, we tested whether p65 is associated with CaMK in vivo. To this end, p65 antibody-directed immunoprecipitates from lysates of HeLa cells transfected with either p65 or p65 plus CaMKIV were utilized as a source for kinase to phosphorylate GST alone, GST/CaMKIV, and GST/CaMKIVc as substrates. In these experiments, GST/p65C was not GST alone was labeled with [γ-32P]ATP only in the presence of CaMK-activating CaM when using cells transfected with p65 alone (Fig. 2C, compare lanes 1 and 2). Similarly, HeLa cells cotransfected with both p65 and CaMKIV exhibited a detectable, basal level of phosphorylation with GST/p65C (Fig. 2C, lane 3), which was further strengthened with addition of CaM (Fig. 2C, lane 4). In particular, phosphorylation of GST fusion to the full-length p65 was readily detectable under this condition (Fig. 2C, lanes 5 and 6). Thus, the putative proteins or signaling events required for efficient phosphorylation of the full-length p65 in vivo may exist in this p65 antibody-directed immunoprecipitate. Notably, CaMKIV is known to be expressed only in certain cell types whereas CaMKI and CaMKII are ubiquitous in expression. Thus, the CaMK found to be associated with p65 in vivo in the absence of cotransfected CaMKIV (Fig. 2C, lane 2) is likely to be either CaMKI or CaMKII. Overall, these results strongly demonstrate that the endogenous CaMK in HeLa cells or cotransfected CaMKIV specifically associates with and phosphorylates p65 in vivo.

Recruitment of CBP and Release of SMRT by CaMKIV-phos-
Phosphorylated p65—The fact that the CaMKIV-directed phosphorylation site was mapped to the C-terminal transactivation domain of p65, which was previously shown to be the interaction interfaces of both transcription coactivator CBP and corepressor SMRT/N-CoR (7, 8, 16), led us to test whether CaMKIV-directed phosphorylation of p65 affects its interactions with these transcription cofactor molecules. Indeed, the mammalian two hybrid-based assays demonstrated that coexpression of CaMKIVc strengthened the interactions of Gal4/p65 with CBP-A or SMRT-D under any condition. Overall, these results clearly demonstrate that CaMKIV-mediated phosphorylation of p65 results in efficient recruitment of transcription coactivator CBP while repulsing corepressor SMRT.

CaMKIV-mediated Translocation of SMRT—Recently, CaMK signaling was shown to promote myogenesis by disrupting MEF2-HDAC complexes and stimulating HDAC nuclear export (26, 27). Similarly, phosphorylation of SMRT by MEKK-1 was shown to inhibit the ability of SMRT to physically tether to its transcription factor partners and led to a redistribution of the SMRT protein from a nuclear compartment to a more perinuclear or cytoplasmic compartment (24). These results were recapitulated in the in vitro GST pull down assays. Radiolabeled CBP-A interacted with p65C, which was reacted with CaM-activated CaMKIVc, but not with CaMKIV alone and washed extensively prior to being added to the GST pull down assays (Fig. 3B). Consistent with the mammalian two hybrid tests, interactions of unphosphorylated GST/p65C and radiolabeled SMRT-D were lost when p65C was phosphorylated by CaM-activated CaMKIVc (Fig. 3B). As expected, GST alone did not interact with radiolabeled CBP-A or SMRT-D under any condition. Overall, these results clearly demonstrate that CaMKIV-mediated phosphorylation of p65 results in efficient recruitment of transcription coactivator CBP while repulsing corepressor SMRT.

CaMKIV-mediated Translocation of SMRT—Recently, CaMK signaling was shown to promote myogenesis by disrupting MEF2-HDAC complexes and stimulating HDAC nuclear export (26, 27). Similarly, phosphorylation of SMRT by MEKK-1 was shown to inhibit the ability of SMRT to physically tether to its transcription factor partners and led to a redistribution of the SMRT protein from a nuclear compartment to a more perinuclear or cytoplasmic compartment (24). These results were recapitulated in the in vitro GST pull down assays. Radiolabeled CBP-A interacted with p65C, which was reacted with CaM-activated CaMKIVc, but not with CaMKIV alone and washed extensively prior to being added to the GST pull down assays (Fig. 3B). Consistent with the mammalian two hybrid tests, interactions of unphosphorylated GST/p65C and radiolabeled SMRT-D were lost when p65C was phosphorylated by CaM-activated CaMKIVc (Fig. 3B). As expected, GST alone did not interact with radiolabeled CBP-A or SMRT-D under any condition. Overall, these results clearly demonstrate that CaMKIV-mediated phosphorylation of p65 results in efficient recruitment of transcription coactivator CBP while repulsing corepressor SMRT.
Transactivation of the NFκB transactivation by CaMKIV. CV-1 or HeLa cells were transfected with LacZ expression vector and various reporter genes and expression vectors, as indicated. CaMKIc (the CaMK residues 1–295) and CaMKIIc (the CaMKII residues 1–290) are the C-terminal deletion mutants with constitutive kinase activities (1), like CaMKIVc (C). ion indicates CaMK activator ionomycin (0.12 μM), and KN denotes CaMK inhibitor KN-93 (3 μM and 30 μM, respectively) (D). 25 ng of each CaMK was cotransfected in C and D. Normalized luciferase expressions from triplicate samples were calculated relative to the LacZ expressions, and the results were expressed as -fold activation (n-fold) over the value obtained with a reporter alone. The data are representative of three similar experiments, and the error bars are as indicated.

![Graph 1](image1)

**Graph 1**: Stimulation of NFκB transactivation by CaMKIV. CV-1 or HeLa cells were transfected with LacZ expression vector and various reporter genes and expression vectors, as indicated. CaMKIc (the CaMK residues 1–295) and CaMKIIc (the CaMKII residues 1–290) are the C-terminal deletion mutants with constitutive kinase activities (1), like CaMKIVc (C). ion indicates CaMK activator ionomycin (0.12 μM), and KN denotes CaMK inhibitor KN-93 (3 μM and 30 μM, respectively) (D). 25 ng of each CaMK was cotransfected in C and D. Normalized luciferase expressions from triplicate samples were calculated relative to the LacZ expressions, and the results were expressed as -fold activation (n-fold) over the value obtained with a reporter alone. The data are representative of three similar experiments, and the error bars are as indicated.

**Stimulation of the NFκB Transactivation by CaMKIV**—The above results suggest that CaMKIV should serve as an activator of the NFκB transactivation. This prediction was confirmed in experiments in which Gal4/p65-directed transactivation of the Gal4-Luc reporter construct (9, 16) was significantly enhanced by coexpressed CaMKIVc in a dose-dependent manner (Fig. 5D). As already noted, the ionomycin effect on the p65 transactivation in HeLa cells non-transfected with CaMKIV is probably because of the endogenous CaMKI. Accordingly, the inhibition was relatively minor with 3 μM KN-93, the concentration that is known to specifically inhibit CaMKII, whereas 30 μM KN-93, at which both CaMKI and CaMKIV are inhibited, completely blocked the p65 transactivation (Fig. 5D). From these results, we concluded that CaMKI and CaMKIV are capable of stimulating the NFκB transactivation, likely through direct phosphorylation of p65 and other proteins that appears to result in efficient recruitment of transcription coactivator CBP while disrupting the p65-SMRT interactions and stimulating nuclear export of corepressor SMRT.

Several reports have demonstrated that inhibition of CaMK activity is associated with apoptosis and proliferation. Inhibition of CaMK activity with specific inhibitors induces apoptosis in NIH 3T3 cells (28) and sensitizes etoposide-resistant cells to apoptotic challenge (29). Thymic T cells from transgenic mice expressing a catalytically inactive form of CaMKIV showed defects in survival and proliferation (30). Similarly, CaMK inhibitor KN-62 was shown to reduce DNA synthesis in small cell lung carcinoma (31). It is interesting to note that NFκB has also been linked to both anti-apoptosis and pro-proliferative activities (reviewed in Refs. 32 and 33). Thus, NFκB may mediate these previously characterized anti-apoptotic and pro-proliferative effects of CaMKIV, although other activities of CaMKIV previously known and/or yet to be characterized may also turn out to be operative through NFκB. Similar to the work described in this report, p65 was recently shown to be phosphorylated by the 1κB-associated PKAc subunit through a cyclic AMP-independent mechanism, which promoted a novel bivalent interaction of p65 with the coactivator CBP/p300 (34, 35). Thus, p65 appears to be directly phosphorylated by at least two distinct kinases. Finally, the C-terminal transactivation domain of p65 is noted to have a numerous number of phosphorylation sites, including consensus CaMK sites [(R/K)XX(S/T)] (1).
In conclusion, we have shown that CaMKIV specifically interacts with and phosphorlates the NFκB component p65, which results in augmented transriptional activity of p65 by facilitated dissociation of SMRT with concomitant, enhanced recruitment of CBP. CaMKIV also stimulated the nuclear export of SMRT. Overall, these results suggest that the NFκB component p65 may serve as a novel downstream phosphorylation target of CaMKIV and act as one of its effector molecules in vivo.

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