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Synergistic activation of the CMV promoter by NF-κB P50 and PKG

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Received 18 June 2004

Abstract

Several DNA binding NF-κB subunits are substrates for cGMP-dependent kinase (PKG) and their transactivation from cognate sites is induced by phosphorylation. This includes p50, which does not have a transcriptional activation domain and therefore needs to bind to other proteins to mediate gene expression. Here, we describe the synergistic transactivation by p50 and PKG from the CMV promoter. This is caused not only by phosphorylation of p50, leading to increased DNA binding, but also by PKG-dependent activation of CRE sites in the promoter. One of the CRE sites is located directly adjacent to a NF-κB site and is essential for p50-mediated induction of transcription. According to the binding of CREB to p50 in pull-down assays and according to the inhibition of p50-dependent transactivation by dominant-negative CREB, this reflects the formation of a transcription factor complex containing CREB and p50. The nuclear translocation of NF-κB is insufficient to distinguish among the multitude of promoters that harbor cognate recognition sites. The phosphorylation of multiple transcription factors by an upstream kinase, such as PKG, can lead to the formation of transcription factor complexes and differential transactivation from a subset of NF-κB sites. These interactions may be relevant for the activation of viral gene expression.
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Keywords: Transcription; CMV; Kinase; Reporter assay; CRE site; NF-κB

The transcription factor NF-κB is typically induced in host defenses after insult and regulates the expression of a large number of genes involved in immune and inflammatory responses. It also plays an important role in antiviral reactions via interferon gene induction. Through adaptation, many viruses exploit the stress responses by the host to enhance their own gene expression. The promoter and enhancer regions for various viral genes contain recognition sites for NF-κB. Specifically, the existence of NF-κB recognition sites in the promoter/enhancer of the immediate early gene of HCMV has been well documented [1].

PKG is a kinase that participates in the regulation of various signal transduction pathways. It was shown to be localized in the cytosol and nucleus, which implies a potential role in the regulation of gene expression. While PKG may prevent the expression of certain genes through the inhibition of RHO or its downstream targets [2,3], it can induce the expression of other genes via activation of CREB [4], NF-κB [5], or TFII-I [6]. This typically occurs through phosphorylation and activation of these transcription factors. Although the induction of PKG enzyme activity occurs in response to stresses, including shear stress in endothelial cells [7] and superantigen stimulation in T-lymphocytes [8], it has not been shown to participate in viral gene expression. Multiple CRE sites [9,10] are present in the HCMV immediate early gene promoter/enhancer (Fig. 1). They may be sensitive to the activation of PKG and ensuing phosphorylation and activation of the CRE binding protein CREB.

We previously studied the phosphorylation of NF-κB p49/52, p50, and p65 by PKG [5]. We found that

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.101

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5' GCGCGCGTTG ACATTGATTA TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG
   TTCATAGCCC ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT
   GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC ATAGTAACGC
   CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT ACGGTAAACT GCCCACTTGG
   CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT TGACGTCAAT GACGGTAAAT
   GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA CTTTCCTACT TGGCAGTACA
   TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT TTGGCAGTAC ATCAATGGGC
   GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA CCCCATTGAC GTCAATGGGA
   GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG TCGTAACAAC TCCGCCCCAT
   TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCTCT-3'
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Fig. 1. Transcription factor binding sites in the CMV promoter. The promoter/enhancer sequence of the human cytomegalovirus IE1 gene is part of the pCR3.1 vector (bases 550–1864 of GenBank sequence X03922). It contains three NF-κB consensus binding sites (GGGACTTTCC, gray underlined sequences) and four CRE sites (TGACGTCA, gray italic sequences). One NF-κB site is immediately adjacent to a CRE site (boxed). There is also one NF-κB site akin to the site in the human Igκ (GGGGATTTCC, underlined).

PKG-dependent phosphorylation enhances the transactivation by NF-κB subunits from their cognate sites and acts as a proximal signal transducer in the activation of NF-κB. It is not known whether the PKG-regulated transcription factors NF-κB and CREB synergize. Their cooperativity in transactivating CMV genes is a distinct possibility because of the presence of multiple cognate sites for both transcription factors in the CMV immediate early gene promoter/enhancer. This could be of importance for gene expression induced by NF-κB p50, which does not have a transactivation domain. It can potentially interact with CREB to activate the CMV promoter/enhancer. Here we further investigate these interactions.

Materials and methods

Reagents. To generate a CMV-driven luciferase reporter (pCR3.1-luc), we digested the luciferase gene from pNF-κB-luc (Clontech) with *Hin*dIII and *Xho*I, purified the fragment, and ligated it into the corresponding restriction sites of the vector pCR3.1. We introduced point mutations in the three NF-κB consensus sites (compare Fig. 1) in pCR3.1-luc by PCR cloning with the Quickchange site directed mutagenesis kit (Stratagene), using the primers 5′-CATAGTAACGCCAATACTCACTTTCCATTGACGTCA-3′, 5′-AGTACATGACCTTATCTCACTTTCCTACTTGGCAGT-3′, and 5′-CACCAAAATCAACCTCACTTTCCAAAATGTCGTAAC-3′ (the mutated bases are underlined). This reporter plasmid was designated pκBmut-luc. The corresponding mutations were also generated in pCR3.1 without the luciferase gene and designated as pκBmut. To generate deletion mutants of the four CRE sites in the CMV sequence in pCR3.1-luc, we used the following antisense primers: 5′-CGACCCCCGCCCATTGCAATAATGACGTATGT-3′, 5′-CAATAGGGACTTTCCATTGCAATGGGTGGACT-3′, 5′-AAGTACGCCCCCTATTGCAATGACGGTAAATG-3′, and 5′-AGTCTCCACCCCATTGCAATGGGAGTTGTTTT-3′ (the deleted core bases ACGT are between the underlined bases). This resulted in the plasmid pCREmut-luc. The reporter plasmid pCREM2-luc was designed to contain a deletion mutation only of the CRE site that is directly adjacent to a NF-κB site (Fig. 1, boxed sequence). The accuracy of the mutations was confirmed by DNA sequencing. A luciferase reporter containing the C-reactive protein promoter (pC/EBP-luc) was generously provided by D. Samols.

DNA expression constructs. To clone mouse PKG Iα cDNA, its message was amplified from frozen mouse kidney with the primers 5′-AGCATGGGCACCCTGCGGGATTTA-3′ and 5′-ATTAGAAGTCTATGTCCCAGCCTGAGTTG-3′. The product was cloned into the vector pCR3.1 (Invitrogen, Carlsbad, CA) followed by subcloning into the vector pEF6/HisB (Invitrogen, Carlsbad, CA). Sequence fidelity and accurate reading frame were verified by DNA sequencing analysis [5]. The plasmids containing human p50 and p65 were obtained from the NIH AIDS Reagent Repository. A-CREB is a dominant-negative inhibitor of CREB, which contains a designed acidic amphipathic extension at the N-terminus of the CREB leucine zipper domain. This extension interacts with the basic region of CREB and thus prevents the basic region of wild-type CREB from binding to DNA [11]. The expression vector for A-CREB was generously provided by C. Vinson and the insert was subcloned into pEF6/HisB using *Bam*HI and *Xba*I.

Electrophoretic mobility shift assay. DNA binding was assessed by electrophoretic mobility shift according to standard protocols [5]. The oligonucleotide used in the gel shift experiments was NF-κB–CRE (a NF-κB site from the CMV promoter sequence, sense 5′-CAA TAG GGA CTT TCC ATT GAC GTC AAT GGG-3′).

Reporter gene assays. Luciferase reporter gene assays were done as previously described [5]. The pCR3.1-luc reporter or its mutant forms were used at 5 ng per transfection. All assays were done at least in triplicate. As confirmation of protein expression, 20 μg of the same lysates was also used for separation on 8% SDS–polyacrylamide gels followed by Western blotting on PVDF membranes.

Pull-down analysis of protein binding. GST-CREB (generously provided by T. Osborne) and GST were expressed in *Escherichia coli* and purified on GSH beads. The binding between NF-κB p50 and CREB was analyzed by pull-down in vitro and in nuclear extracts from transfected 293T cells. In vitro, purified recombinant p50 (Promega) 116 ng/sample, PKG 90 ng/sample (Promega) or p50 plus PKG was incubated in 20 μl kinase reaction buffer (250 mM Mes, pH 6.9, 2 mM EGTA, 5 mM magnesium acetate, 50 mM NaCl, 10 mg/ml BSA, and 100 mM dithiothreitol) with 0.2 mM cGMP for 15 min at room temperature. For precipitation, the volume was increased to 200 μl with LSAB buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, and 2 mM $MgCl_2$). The reaction mix was precleared with 20 μl of 20% bead suspension at 4 °C for 1 h before incubation with 20 μl of beads attached to either GST-CREB or GST at 4 °C for 1 h. For determination of binding after transfection, 400 μg of nuclear extracts from 293T cells transduced with vector, p50, PKG, or p50 plus PKG was precleared with 20 μl of 20% bead suspension at 4 °C for 1 h before incubation with 20 μl of 20% of beads attached either to GST-CREB or GST at 4 °C for 1 h. Following six washes in LSAB buffer, the attached proteins were separated on 8% reducing SDS–PAGE alongside with 5% (nuclear extracts) or 10% (in vitro reaction) of the input material.

Kinase reaction. The enzymatic activity of cGMP-dependent kinase was analyzed as previously described [5].

Cells. The reporter assays were performed by transient transfections of 293T cells.

Results

Transactivation from the CMV promoter by NF-κB p50 depends on a CRE sequence

We previously analyzed the activation of NF-κB p50 by PKG [5]. When we expressed the genes of interest under the control of a CMV promoter construct, we noted that the transfected PKG kinase activity and protein levels were substantially increased by co-transfection of p50. After cloning of PKG into a vector that is not sensitive to NF-κB (pEF6/HisB), the levels of transfected PKG were not affected by NF-κB co-transfection (not shown). This suggested a reciprocal activation between p50 and PKG in the transactivation of the CMV promoter. We designed experiments to identify the mechanism of these interactions.

PKA has been implied in HCMV expression through CREB [12]. PKG also can phosphorylate and activate CREB, but the induction of CMV gene expression by PKG has not been demonstrated. To confirm PKG- or NF-κB-induced transactivation from the HCMV IE1 enhancer/promoter, we co-transfected p50, p65, or PKG with a luciferase reporter under the control of this CMV sequence. Co-transfection of increasing amounts of NF-κB p50 or p65, or of increasing amounts of PKG-mediated increased luciferase readouts (Figs. 2A and B), confirming the functionality of the NF-κB and CRE sites in the CMV promoter/enhancer. Transfection of PKG plus p50 or PKG plus p65 led to stronger transactivation from the CMV promoter reporter than PKG or NF-κB alone, indicating that the interaction was additive or synergistic (Figs. 2A and B). We further analyzed whether PKG and NF-κB act mutually independently on CMV by mutating all NF-κB consensus sites or all CRE sites in the reporter construct. In the absence of NF-κB consensus sites in the reporter, no transactivation was detected by p65 or p50. In contrast,

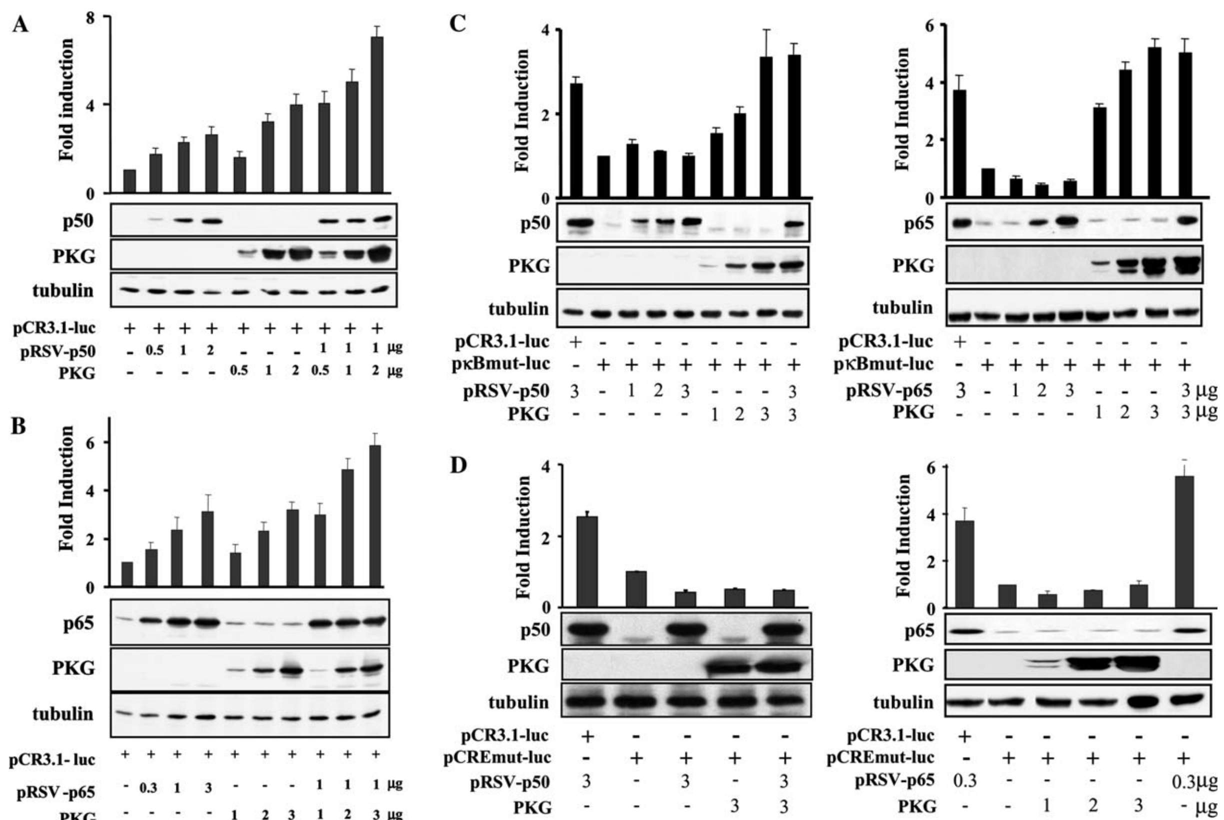

Fig. 2. Transactivation of a CMV-driven reporter gene by NF-κB and PKG. The luciferase gene was cut from the pNF-κB-luc reporter by *Hin*dIII and *Xba*I, and subcloned into the pCR3.1 vector, which contains the CMV promoter (designated as pCR3.1-luc). 293T cells were transiently transfected with 5 ng pCR3.1-luc plus increasing amounts of pRSV-p50, pRSV-p65, or pEF6/HisB-PKG, individually or together. The values are normalized to the luminescence induced by 10 ng of co-transfected *Renilla* construct pRL-SV40 and the results (fold induction) are presented as means ± standard deviation of three replicates (top panel). The protein expression of the transfected molecules correlated with the amounts of DNA introduced into the cells, while tubulin (used as a loading control) remained constant (bottom panel). (A) Transactivation from the CMV promoter/enhancer induced by increasing amounts of p50, PGK, or p50 plus PKG. (B) Transactivation from the CMV promoter/enhancer induced by increasing amounts of p65, PKG, or p65 plus PKG. (C) Transactivation from a reporter with inactivating mutations in all three NF-κB consensus sites (left panel: p50 and PKG, right panel: p65 and PKG). (D) Transactivation from a reporter with deletion mutations of all four CRE sites (left panel: p50 and PKG, right panel: p65 and PKG).

transfected PKG dose-dependently induced luciferase activity from the CMV promoter construct, regardless of whether the NF-κB sites were mutated or intact (Fig. 2C). Mutation of all the four CRE sites in the reporter construct abrogated the PKG-induced transactivation. Co-transfection of p65 mediated luciferase activity regardless of the mutation of the CRE sites, however, the luciferase activity mediated by p50 was lost (Fig. 2D).

One of the NF-κB sites is located directly adjacent to a CRE site (boxed sequence in Fig. 1), raising the possibility that the cognate transcription factors form a complex. Because p50 does not have a transactivation domain, homodimers of p50 are thought to mediate transcriptional repression, however, its potential association with CREB could account for p50-induced reporter activity. We therefore mutated only the CRE site that is directly adjacent to a consensus NF-κB site in the CMV promoter/enhancer reporter construct. Expectedly, while transactivation from this construct by p65 was unaffected and transactivation by PKG was only moderately reduced, the mutation of this one CRE site was sufficient to abrogate the transactivation driven by p50. It also prevented the p50-dependent amplification of the reporter activity induced by co-transfected PKG (Fig. 3A). These results suggested that the transactivation of the HCMV promoter/enhancer by p50 is stringently dependent on an interaction between p50 and a CRE binding protein.

A-CREB is a dominant-negative mutant of CREB [11]. We asked whether this construct could inhibit the p50-mediated transactivation of the CMV reporter. Expectedly, the co-transfection of A-CREB into 293T cells suppressed the reporter activity induced by PKG. Furthermore, A-CREB co-transfection inhibited the transactivation induced by transfected p50 or p50 plus PKG (Fig. 3B). Therefore, CREB may be essential for the induction of CMV gene expression by p50.

PKG facilitates the binding between p50 and CREB

We tested by pull-down assay whether a complex can be formed between p50 and CREB. Initially, we incubated purified p50 and PKG in a kinase reaction, with p50 or PKG alone serving as controls. We performed pull downs with GST-CREB beads [13] or GST beads. GST-CREB was able to bind to PKG, but only modestly to p50. Following the incubation of PKG plus p50, the amounts of pulled-down PKG and p50 increased (Fig. 4A). We then precipitated CREB-binding proteins from nuclear extracts of 293T cells transfected with p50, PKG, or p50 plus PKG. Visible amounts of p50 were

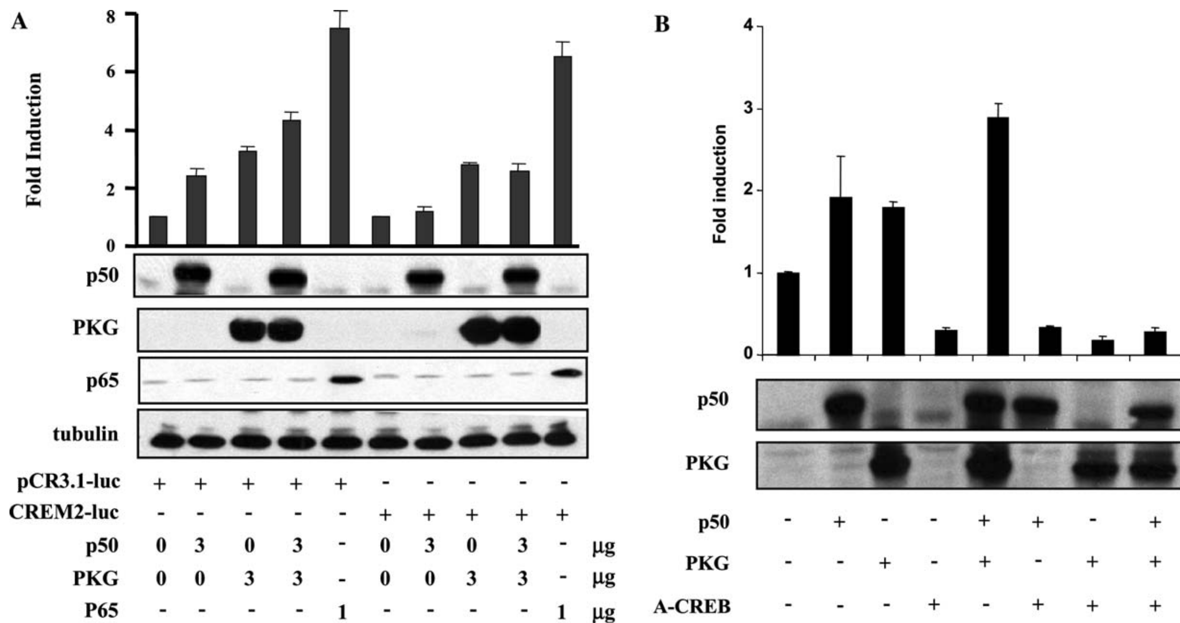

Fig. 3. The NF-κB site adjacent to a CRE site is critical for p50-dependent transactivation. (A) Mutation of one critical CRE site abrogates transactivation by p50. The one CRE site that is directly adjacent to a NF-κB site in the CMV promoter/enhancer sequence was mutated in the CMV-driven luciferase reporter construct. The reporter was designated as pCREM2-luc. 293T cells were transiently transfected with 5 ng pCREM2-luc (or pCR3.1-luc as positive control) plus pRSV-p50 or pEF6/HisB-PKG. The values are normalized to luminescence induced by 10 ng co-transfected Renilla construct RL-SV40 and the results (fold induction) are presented as means ± standard deviation of three samples (top panel). Western blotting confirmed the expression levels of the transfected molecules. (B) Co-transfection of A-CREB suppresses p50 transactivation. 293T cells were plated at 1×10^6 in 100 mm diameter petri dish and grown for 24 h before transfection with $CaCl_2$. Five nanograms of pCR3.1-luc plus 20 ng pRL-TK-luc (internal control) was cotransfected with p50, PKG, A-CREB alone or in combination. Twenty-four hours later, the cells were harvested in 1 ml reporter lysis buffer (Promega). Lysates were diluted 1:40 and 10 µl was used for dual luciferase reporter assay. The expression of the transduced proteins was confirmed by Western blotting.

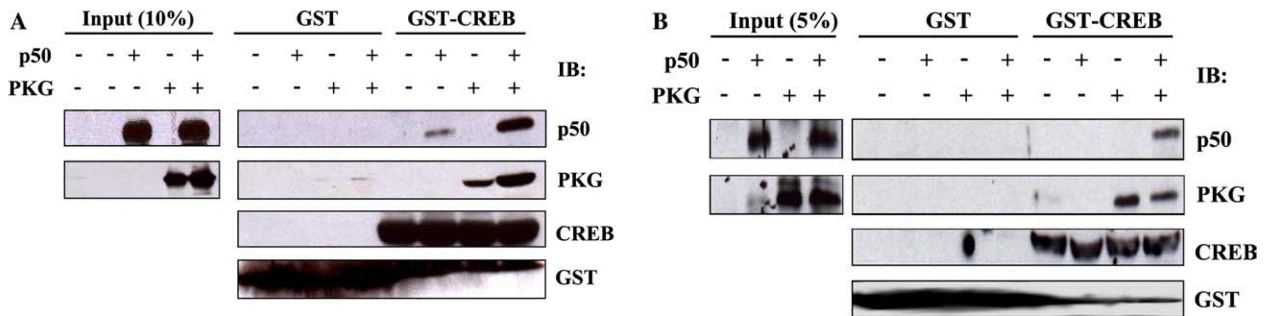

Fig. 4. Binding of p50 and CREB is enhanced by PKG. (A) Pull-down with purified proteins. One hundred sixteen nanograms per sample purified p50 was incubated with or without purified PKG for 15 min at room temperature. Twenty microliters of 20% GST-CREB or GST was added to the reaction solution and incubated at 4 °C for 1 h with gentle shaking. Following six washes with LSAB buffer, the bound proteins were analyzed by SDS–PAGE followed by Western blotting. (B) Pull-down from nuclear extracts. 293T cells were transfected with p50, PKG, p50 plus PKG, or vector controls. Nuclear extracts were prepared 48 h after transfection. Four hundred micrograms of protein was precleared and then incubated with 20 μl of 20% GST-CREB or GST beads for 1 h at 4 °C. Following six washes with LSAB buffer, bound proteins, compared to 5% of the input, were separated by SDS–PAGE and analyzed by Western blotting. IB, immunoblot. The trailing signal of GST reflects its migration in the dye front on 8% SDS–PAGE. The results are representative of two experiments.

seen on the Western blots only in the sample transfected with p50 plus PKG (Fig. 4B). According to these results, the observed interaction may be based on increased affinity between p50 and CREB following phosphorylation by PKG. Alternatively, it may represent the formation of a tri-molecular complex containing PKG, p50, and CREB (Figs. 4A and B).

PKG enhances the binding by p50 to the NF-κB–CRE sequence

Changes in transactivating activity may reflect alterations in DNA binding. PKG phosphorylates NF-κB proteins and induces the binding of p65 to the NF-κB consensus sequence (data not shown) as well as the binding of p50 to the non-canonical NF-κB site CATA GTGGCGCAAACTCCCTTACTGC (NF-κB site underlined) [5]. We therefore tested the binding of p50 to an oligonucleotide that represents the NF-κB sequence adjacent to the CRE site in the CMV promoter/enhancer CAATAGGGACTTTCCATTGACGTCA ATGGG (NF-κB site underlined). Phosphorylation by cGMP-dependent kinase, followed by the transfer of p50 from kinase assays to gel shift reaction mixtures, induced the binding of p50 to the NF-κB–CRE sequence in vitro. After co-transfection, the binding affinity by nuclear extracts to the same probe was similarly induced and the specificity of the main DNA binding band from transfectants of p50 or p50 plus PKG was confirmed by supershift (Figs. 5A and B).

NF-κB sites in the CMV promoter compete with other NF-κB sites for the transcription factor

We reasoned that the presence of four NF-κB sites in the CMV promoter sequence would likely compete with other NF-κB sites for the binding of p65 or p50. When

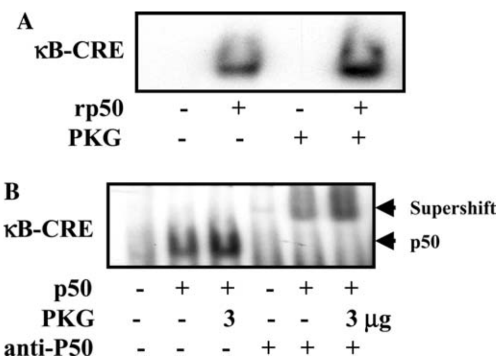

Fig. 5. PKG enhances DNA binding by NF-κB p50. PKG-dependent activation of p50 induces its affinity to oligonucleotides that represent the CRE-proximal NF-κB sequence contained in the CMV promoter (κB-CRE). (A) Twenty-five nanograms of recombinant p50 per sample was phosphorylated at room temperature for 15 min by 1 U purified PKG with or without 200 μM cGMP in total volume of 10 μl. The reaction mixtures were transferred to DNA binding buffer and incubated for additional 20 min with the indicated ^{32}P-labeled probe. The reactants were resolved on native 4% polyacrylamide gels and exposed to autoradiography film. (B) 293T cells were transiently transfected with 0.3 μg pRSV-p50 with or without 3 μg pEF6/HisB-PKG. Twenty-four hours after transfection, the cells were harvested, washed in PBS, and nuclear extracts were prepared. 10 μg of nuclear protein was used for electrophoretic mobility shift assay with radiolabeled κB-CRE oligonucleotides. The identity of the major DNA-binding band was confirmed by supershift with 50 ng anti-p50 antibody, added to the nuclear extracts at room temperature for 10 min before DNA binding.

we used the pEF6/HisB vector to adjust the total amount of transfected DNA to 10 μg, transactivation from the C/EBP reporter by co-transfected p50 (Figs. 6A and B) or from the NF-κB reporter by co-transfected p65 (Figs. 6C and D) was efficient. In contrast, when we used increasing amounts of the CMV containing vector for adjustment of the total amount of DNA, the luciferase activity declined dose-dependently (Figs. 6B and D). This inhibition was not seen with a CMV vector, in

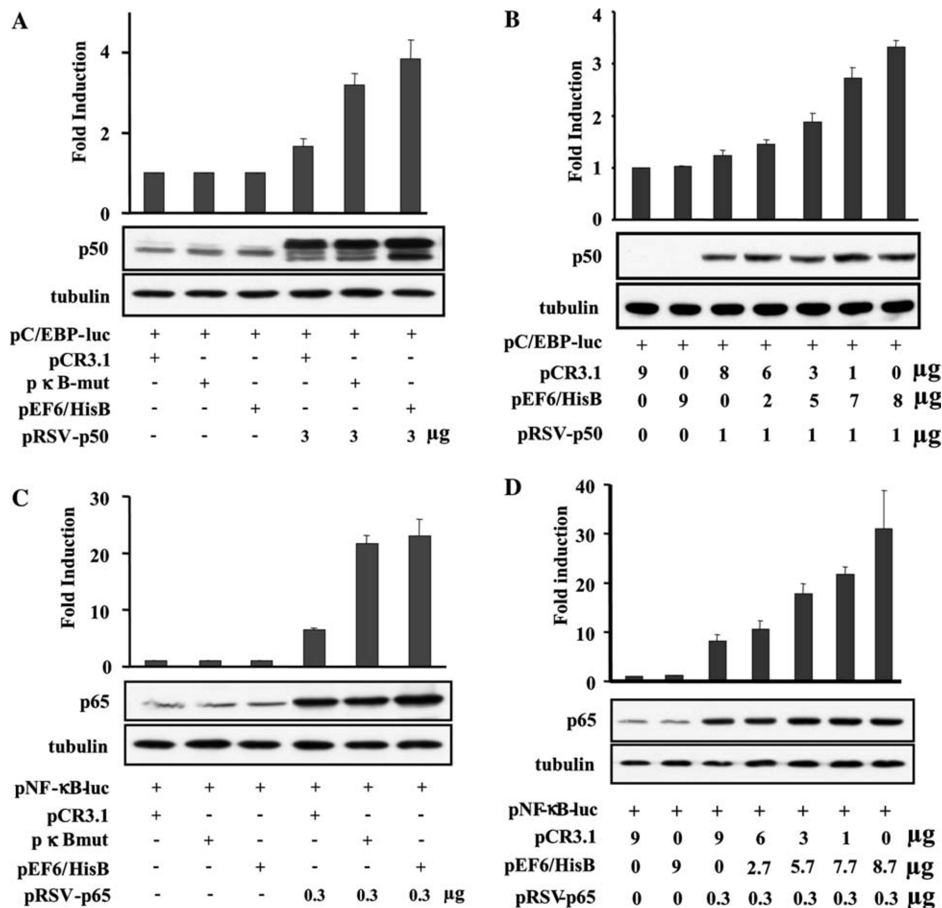

Fig. 6. The presence of CMV promoter/enhancer DNA affects NF-κB transactivation. (A) 293T cells were transiently transfected with 1 μg pC/EBP-luc with or without 3 μg pRSV-p50. To test the effect of excess NF-κB sites on p50-dependent transactivation, pCR3.1, pκBmut (mutations in all three consensus NF-κB sites in the CMV promoter/enhancer region), and the non-CMV vector pEF6/HisB were used to balance the total amount of DNA for transfection. After 24 h, the cells were harvested in reporter lysis buffer. Forty micrograms of lysate was used for luciferase measurement by luminometer and the values obtained for the vector control group were normalized to 1. The results represent means ± standard deviation of triplicate samples. (B) 293T cells were transiently transfected with 1 μg pC/EBP-luc plus 1 μg pRSV-p50. Various ratios of pCR3.1 vector and pEF6/HisB were used for balancing the total amount DNA to 10 μg. The luciferase assay was carried out as in (A). (C) 293T cells were transiently transfected with 0.3 μg pNF-κB-luc with or without 0.3 μg pRSV-p65 plus 10 ng pRL-SV40. To determine the effect of NF-κB sequences on p65 transactivation, pCR3.1, pκBmut, and the non-CMV vector pEF6/HisB were used for balancing the total amount of DNA for transfection. Twenty-four hours after transfection the cells were harvested in 1 ml reporter lysis buffer. The lysates were analyzed at 1:40 dilution for luciferase activity and the values obtained for the vector control group were normalized to 1. The results represent means ± standard deviation of triplicate samples. (D) 293T cells were transiently transfected with 0.3 μg pNF-κB-luc with or without 0.3 μg pRSV-p65 plus 10 ng pRL-SV40. Various combinations of pCR3.1 vector and pEF6/HisB vector were used for balancing the total amount of DNA of transfection. The luciferase assay was carried out as in (C).

which all three NF-κB consensus sites had been mutated (Figs. 6A and C). Hence, the presence of NF-κB binding sites within the nucleus may impact the readout of reporter activity.

Discussion

We have previously shown that PKG can phosphorylate NF-κB p50 and enhance its DNA binding and transactivation from a non-consensus sequence [5]. Here we demonstrate the functional interaction of CREB and p50 on the level of transactivation. It reflects a complex formation between p50 and CREB that is facilitated by PKG. This is in keeping with the recent demonstrations that activated NF-κB may interact with the CRE Binding Protein [14,15]. Alternatively, C/EBP (CCAAT Enhancer Binding Protein) can bind to the CRE site [16,17] and synergize with p50 in inducing gene expression. However, this is less likely to play a role here because C/EBP has been reported to inhibit transactivation from the CMV promoter/enhancer [18]. The binding between p50 and CREB implies multiple levels of interactions between PKG and p50, which lead to gene expression. First, p50 is a substrate for PKG and its phosphorylation enhances its DNA binding. Second, p50-mediated transactivation from a cognate site can be amplified by binding of the PKG substrate CREB

to an adjacent CRE site. These interactions may also bear on HTLV infection because Tax transactivation proceeds via CREB and NF-κB [19].

A large number of gene promoters and enhancers contain recognition sites for NF-κB. While the release of p65/p50 into the nucleus is insufficient to differentially regulate transactivation from those sites, additional biochemical mechanisms have been identified that control which of the genes driven by NF-κB sequences are activated at any particular time. One such mechanism is the formation of complexes of NF-κB subunits with transcription factors that recognize adjacent promoter sequences [15,18,20]. In particular, the p50 subunit of NF-κB does not have a transactivation domain and the induction of gene expression by p50 depends on its interaction with suitable binding partners. Besides p65, the transcription factors BCL-3 [21], TFII-I [22], and C/EBP [20] have previously been identified to serve in these roles. Such interactions may account for the differential effects between p65 and p50 on the gene expression of some cytokines [23]. p50 functions that are independent of p65 may also explain the importance of the p50 subunit in the regulation of apoptosis [24]. Viral genes can be differentially transactivated by p65 versus p50. This has been demonstrated for homodimers of p50 or p52, which in conjunction with TFII-I assemble a preinitiation complex in the transcription of HIV-1 [22]. The transactivation of HCMV gene expression by p50 plus CREB is also a mechanism of differential regulation by NF-κB p65 and p50.

Recent evidence has demonstrated that gene expression is typically regulated by multi-subunit transcription factor complexes [25–27]. The mechanisms that regulate the assembly of these composites are incompletely understood. The present study is an example of the activation of several member proteins of a transcription factor complex, here NF-κB p50 and CREB, by one single kinase, here PKG. In a different mode of NF-κB activation, PKA can phosphorylate p65 and promote its interaction with CBP/p300 [14,15]. This suggests the existence of two distinct modes of NF-κB activation, driven by kinases that promote the assemblies of different transcription factor complexes.

One approach to the analysis of gene functions is their forced over-expression in specific cell lines. Because viral promoters induce very high levels of gene expression, they are considered first choice in transfection assays. Although transactivation studies of NF-κB have often involved the co-transfection of genes of interest under the control of the CMV promoter, skewing of the results by interactions between NF-κB proteins and CMV promoter sequences is a distinct possibility. Our experiments highlight potentially confounding factors in transient transfection assays with reporter genes, in particular when performed with plasmids containing highly active viral promoters, such as CMV. Among various samples within the same experiment, the total amount of transfected DNA is typically kept constant by balancing with the CMV containing vector. The NF-κB binding sites in the transfected CMV promoters can compete with the NF-κB sites in the reporter constructs. This leads to a profound reduction of the reporter signal, in our experiments up to 75%. Furthermore, NF-κB proteins, such as p50, can alter the expression levels of co-transfected genes by transactivation from one of the NF-κB sites in the CMV promoter. The present investigations have revealed the potential for a reciprocal influence between the co-transfected kinase and p50 under these circumstances, which interfered with the quantitation of phosphorylation-induced changes in p50-dependent transactivation. This was further complicated by four CREB sites that mediated PKG-dependent transactivation. One of these CREB sequences is adjacent to a NF-κB site in the CMV promoter and is essential for the p50-induced transactivation. For accurate measurements, we were forced to re-clone both PKG and NF-κB genes into vectors that are not sensitive to either set of transcription factors. Although reporter assays are currently often done with constructs that contain CMV promoter sequences, these sources of interference need to be taken into account during the performance of these types of assays.

Acknowledgments

This study was supported by National Institutes of Health research Grant CA76176 to G.F.W. The plasmids containing human p50 and p65 were obtained from the NIH AIDS Reagent Repository. A luciferase reporter containing the C-reactive protein promoter (pC/EBP-luc) was generously provided by D. Samols (Department of Biochemistry, Case Western Reserve University, Cleveland, OH, USA). The A-CREB construct was a kind gift from C. Vinson (National Institute of Health, Bethesda, MD, USA). We are grateful to T.F. Osborne (Department of Molecular Biology and Biochemistry, University of California, Irvine, CA, USA) for providing the GST-CREB construct.

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