DNA Binding of Repressor Nuclear Factor-κB p50/p50 Depends on Phosphorylation of Ser337 by the Protein Kinase A Catalytic Subunit*

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The NF-κB p50/p50 homodimer is mainly associated with transcriptional repression. Previously, we demonstrated that phosphorylation of NF-κB p50 Ser337 is critical for DNA binding. Here, we report that p50 Ser337 is constitutively phosphorylated by the protein kinase A catalytic subunit (PKAc) in three different cell types, which may account for the constant binding of p50/p50 to DNA in unstimulated cells. This was demonstrated first by showing that treatment of cells with PKAc-specific inhibitors blocked p50/p50 DNA binding. Second, phosphorylation of p50 by PKAc was prevented by substitution of Ser337 to alanine. Third, both p50 and PKAc proteins as well as kinase activity that phosphorylates p50 were found to co-fractionate following gel filtration chromatography. Finally, PKAc and p50 were shown to be able to reciprocally co-immunoprecipitate one another, and their physical association was blocked by a PKA catalytic site inhibitory peptide. This indicates that phosphorylation of p50 Ser337 involves direct contact with the PKAc catalytic center. In contrast to the dramatic elevation of nuclear p50/p50 heterodimers induced by tumor necrosis factor α, DNA binding of p50/p50 homodimers was not greatly altered. Taken together, these findings reveal for the first time that there is a direct interaction between PKAc and p50 that accounts for constitutive phosphorylation of p50 Ser337 and the existence of DNA bound p50/p50 in the nuclei of most resting cells. This mechanism of DNA binding by p50/p50 following phosphorylation of Ser337 by PKAc may represent an important means for maintaining stable negative regulation of NF-κB gene expression in the absence of extracellular stimulation.

The nuclear factor NF-κB is a transcription factor identified by Sen and Baltimore nearly 20 years ago (1). NF-κB plays a critical role in transcription regulation of genes involved in immune response, inflammation, cell proliferation, differentiation, apoptosis, and oncogenesis (2–6). In vertebrates, five immune response, inflammation, cell proliferation, differentiation domain termed RHD within the N-terminal 300 amino acid region. The RHD is responsible for DNA binding, dimerization, nuclear translocation, and interaction with IκB inhibitory proteins. Although all NF-κB family members can bind to DNA, only p65, RelB, and c-Rel contain a transactivation domain in their C-terminal regions.

The NF-κB family members can form various homodimers and heterodimers among which the p50/p65 heterodimer is the most abundant and studied species. In most resting or unstimulated cells, p50/p65 heterodimers are confined in the cytoplasm in an inactive form by forming a complex with IκB proteins. Treatment of cells with NF-κB stimuli such as cytokines, mitogens, and bacterial lipopolysaccharide leads to phosphorylation of IκB by IκB kinase and subsequent ubiquitination and degradation of IκB by the 26 S proteasome (7, 8). This allows NF-κB to translocate to the nucleus where it binds to many target promoters and transactivates gene expression.

NF-κB transactivation activity requires phosphorylation of p65 at specific serine residues, which mediates interaction of NF-κB with transcriptional cofactors including CBP/p300 (9, 10). A number of protein kinases have been identified for p65 phosphorylation. In response to lipopolysaccharide and tumor necrosis factor α (TNFα) treatments, protein kinase A catalytic subunit (PKAc) and mitogen- and stress-activated protein kinase-1 (MSK1), respectively, can phosphorylate serine 276 (Ser276) within the RHD of p65 (11, 12). TNFε can also induce phosphorylation of Ser311 by PKCε (13). In addition, casein kinase II and IκB kinase α are able to phosphorylate Ser292 and Ser356, respectively, in the C-terminal transactivation domain (14–16). Other kinases including Ca2+/calmodulin kinase IV and cyclic GMP-dependent kinase have also been reported to phosphorylate p65 in the C-terminal region, although the exact phosphorylation site(s) has not been determined (17, 18). In all of these cases, phosphorylation of p65 resulted in elevated transactivation activity of NF-κB.

Because of the lack of transactivation domains, p50 is considered to be mainly responsible for DNA binding. Indeed, NF-κB DNA binding requires phosphorylation of p50 (19–22). Moreover, phosphorylation of p50 was shown to dramatically enhance the stability of the NF-κB DNA complex (22). In this regard, p50 phosphorylation plays an important role in regulating NF-κB activity. In addition, p50 itself can form p50/p50 homodimers, which generally function as transcriptional repressors by inhibiting transactivation of p50/p65 heterodimers (10, 23–28). In contrast to the cytoplasmic confinement of p50/p65 heterodimers, p50/p50 homodimers are found in the nuclei of most resting cells.

The abbreviations used are: TNFα, tumor necrosis factor α; PKAc, protein kinase A catalytic subunit; MSK1, mitogen- and stress-activated protein kinase-1; ERK, extracellular signal-regulated kinase; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PKI, PKA-specific peptide inhibitor; wt, wild type; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay.

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of most resting cells and are capable of DNA binding (10, 23, 24, 27, 29). This suggests that p50 is phosphorylated in resting cells. However, little is known about how p50 is phosphorylated and what kinase(s) is involved. Previous studies in our laboratory demonstrated that Ser337 of p50 is phosphorylated and is critical for DNA binding (19). Substituting this serine with an alanine dramatically reduced p50/p50 DNA binding activity. We now provide evidence showing that p50 Ser337 is phosphorylated by PKAc constitutively in different cell types. This is the first kinase that has ever been demonstrated to be responsible for p50 phosphorylation. We propose that this phosphorylation is essential to the mechanism by which p50/p50 homodimers maintain transcriptional repression of NF-κB target genes in unstimulated cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa, COS-7, and KAd5 cells were cultured as described previously (19, 30).

Reagents and Antiseras—Recombinant human TNF-α was purchased from BIOSOURCE. H89 was obtained from Alexis Biochemicals. Myristoylated cell-permeable PKA inhibitor 14–22 amide (Myr-PKI), cell-permeable ERK activation inhibitor peptide I (ERKI), and 5,6-dichloro-1-b-ribofuranosylbenzimidazole (DRB) were purchased from Calbiochem. PKI 6–22 amide, KN-62, U0–126, KT 5923, PD98059, SB203580, 8-Br-cAMP, dibutyryl cAMP, and Rp-8-Br-cAMP were purchased from Sigma. Rabbit anti-PKAc catalytic subunit (PKAc, C-20) and goat anti-p50 (C-19), p65 (A-14), and MSK1 (H-19) were purchased from Santa Cruz Biotechnology. Rabbit anti-PKAc antibody NT was acquired from Upstate Biotechnology. Rabbit anti-p50 (#1613), p65 (#1226), and LbHa (#1258) were kind gifts of Nancy Rice. Plasmids—Wild type (wt) p50 cDNA clones pCMV-hp50, pGEX-mp50, and their mutants pCMV-hp50S337A and pGEX-mp50S337A, respectively, were described previously (19). To clone mouse PKAc, cDNA of the gene was amplified by reverse transcription-PCR using primers 5′-TATAAGCTTCCACCATGGGCAACGCCGCCGCCGCC-3′ and 5′-ATATCTAGACTAATAACTCACTGTTCTGCGC-3′. PCR products were then cloned into BamHI and XbaI sites of plasmid pRC/CMV (Invitrogen) and confirmed by sequencing.

Cell Transfection—Transfection of COS-7 was performed using GeneJammer transfection regent (Stratagene) according to the manufacturer’s instructions.

Preparation of Nuclear, Cytoplasmic, and Whole Cell Extracts—Nuclear, cytoplasmic and whole cell extracts were prepared as described previously (19, 21).

In Gel Kinase Assay—200 μg of cytoplasmic proteins or 100 μg of nuclear proteins of HeLa cells was fractionated on a SDS-polyacrylamide gel (10%) containing purified glutathione S-transferase (GST), GST تمامى (residues 249–363) with a serine to alanine mutation at position 337 (S337A) (6), and GST-p50 wt, or mutant peptide (residues 249–363) with a serine to alanine dramatically reduced p50/p50 DNA binding activity. Critical for DNA binding (19). Substituting this serine with an alanine mutation at position 337 (S337A) (6)

Gel filtration column (Superdex 200 HR 10/30, Amersham Biosciences) equilibrated with Tris buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol). The immunoprecipitated complexes and the chromographic fractions were then used for in vitro kinase assay as described (19).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was conducted as described previously (31). A double-stranded oligonucleotide of the MHC class I R1 enhancer region, which contains an NF-κB recognition site, was labeled with [α-32P]dCTP and used as the probe as described elsewhere (19).

RESULTS

Phosphorylation Is Required for DNA Binding of p50/p50 Homodimers in HeLa Cells—p50/p50 homodimers have been found in the nuclei of many unstimulated cells including HeLa cells (10, 23, 24, 27, 29). To test if p50/p50 in the nuclei of HeLa cells is phosphorylated and is able to bind to DNA, nuclear extracts were prepared and analyzed for DNA binding by EMSA. A 32P-labeled double-stranded oligonucleotide of the R1 region of the MHC class I enhancer, which contains a recognition site for both p50/p50 homodimers and p50/p65 heterodimers, was used as a probe for the gel shift assay. DNA binding of only p50/p50 (but not p50/p65) was observed in unstimulated HeLa cells (Fig. 1, lane 1). This was confirmed by the ability to supershift with a p50 antibody but not a p65 antibody (lanes 3 and 4). Consistent with our previous findings that dephosphorylation prevents p50/p50 DNA binding (20, 21), treatment of the HeLa cell nuclear extracts with calf interstitial alkaline phosphatase completely abolished DNA binding of p50/p50 (lane 2). This result suggests that in HeLa cells constitutive phosphorylation of p50 is essential for DNA binding of the homodimer.

Next we examined whether the p50/p50 DNA binding in HeLa cells could be enhanced by cytokine treatment as occurs with the p50/p65 heterodimer. As shown in Fig. 1, the p50/p50 DNA complexes were not significantly affected by treatment with TNFα (lane 5). This is in sharp contrast to the dramatic increase in p50/p65 DNA binding (lane 5) as identified by supershift with both p50 and p65 antibodies (lanes 7 and 8). As expected, calf interstitial alkaline phosphatase treatment ab-
Interestingly, no difference in kinase activities was observed between GST-p50 and the two negative control substrates GST and GST-S337A when nuclear extracts of unstimulated HeLa cells were analyzed (data not shown). These results indicate that p50 Ser337 is constitutively phosphorylated in the cytoplasm.

Several potential kinases could phosphorylate the p50 Ser337 based on the sequence surrounding this residue (LRKKSDEL) as well as the sizes of the kinases determined by the in gel kinase assay. These kinases, with molecular masses of their catalytic subunits indicated, include PKA (~41 kDa), casein kinase II (~44 kDa), Ca2+/calmodulin kinase II (~50 kDa), cyclic GMP-dependent kinase (~85 kDa), and p90 RSK (~90 kDa). Because MSK1 is exclusively located in the nucleus (32), this kinase seems unlikely to be involved in p50 Ser337 phosphorylation. To investigate if p50 is a substrate of any of these kinases that is critical for p50/p50 DNA binding, HeLa cells were treated with chemical inhibitors, and then EMSA analysis was performed. Treatment with KN-62, U0–126, and KT5823, which inhibit Ca2+/calmodulin kinase II, p90 RSK (through the inhibition of mitogen-activated protein kinases) and cyclic GMP-dependent kinase, respectively, had little or no effect on p50/p50 DNA binding (Fig. 3A, lanes 2, 3 and 6 of the upper panel) when compared with the mock treatment (lane 1). Casein kinase II inhibitor DRB blocked p50/p50 homodimer binding but surprisingly promoted...
p50/p65 heterodimer binding (lane 4). H89, a potent inhibitor of PKA, greatly inhibited p50/p50 DNA binding (lane 5). Western blot analysis using the same extracts confirmed the presence of equal amounts of p50 for each binding assay (Fig. 3A, lower panel). Similar results were obtained with KAd5 cells (Fig. 3B), which are mouse BALB/c kidney cells transformed by adenovirus type 5 (32). It is noteworthy that in KAd5 cells, p50 is hyperphosphorylated and NF-κB binds to DNA constitutively (21, 33). Treatment of KAd5 cells with H89 inhibited both p50/p50 and p50/p65 DNA binding activities (Fig. 3B, lane 5). In contrast, there was little or no inhibition effect on NF-κB DNA binding when the other inhibitors were applied (lanes 1–4 and 6). Interestingly, DRB treatment increased p50/p65 heterodimer binding (Fig. 3B, lane 4), which is consistent with the result obtained from HeLa cells.

To further determine the kinase(s) for p50 phosphorylation, p50 plasmids were transfected into COS-7 cells in which the expression of NF-κB proteins is minimal. Following transfection, the cells were treated with the kinase inhibitors mentioned above, and whole cell extracts were then prepared and analyzed by EMSA. Treatment with H89 led to a decreased p50/p50 DNA binding (Fig. 3C, lanes 6 and 7), although DRB also reduced the homodimer binding activity (lane 5). In agreement with the results obtained from HeLa and KAd5 cells, the other reagents exhibited little or no inhibition in p50/p50 DNA binding (lanes 2–4 and 8). Because only H89 consistently inhibited p50 DNA binding activity in the three different cell types tested, it is likely that PKA is the major kinase responsible for p50 phosphorylation.

**PKA Is the Kinase Responsible for ConstitutivePhosphorylation of p50**

—Although the above data indicate that PKA is likely responsible for phosphorylation of p50, we cannot absolutely exclude the possibility that MSK1 is also involved because H89 cannot inhibit both PKA and MSK1. To address this, HeLa cells were treated with cell-permeable myristoylated PKA-specific inhibitory peptide (Myr-PKI), which contains a serine to alanine substitution within the consensus sequence for PKA substrates. Because the ERK signaling pathway is involved in MSK1 activation (32), HeLa cells were also treated with cell-permeable ERK inhibitory peptide (ERKI). As seen in Fig. 4, constitutive DNA binding of p50/p50 was blocked by Myr-PKI in HeLa cells (lanes 2 and 3). In contrast, the DNA binding activity was not affected by ERKI (lane 4) or mock treatment (lane 1). Furthermore, when HeLa cells were treated with a combination of inhibitors SB203580 and PD98059, which completely inhibit MSK1 by blocking the p38 and ERK signaling pathways, respectively (11), no change was observed for p50/p50 DNA binding (data not shown). These results strongly indicate that PKA, rather than MSK1, is the kinase responsible for p50 phosphorylation.

To demonstrate that cellular PKA can directly phosphorylate p50, we performed an in vitro kinase assay. PKA was immunoprecipitated from HeLa and KAd5 cells and then subjected to in vitro kinase assay using GST-p50 wt or S337A mutant peptides (residues 249–363) as substrates. Only the wt p50 (lanes 1 and 3) but not the mutant p50 (lanes 2 and 4) was phosphorylated. B. lysates of HeLa cells were fractionated through a gel filtration column. Fractions were tested for kinase activity using GST-p50 wt 249–363 peptides as substrate. The same fractions were also analyzed by Western blot using the indicated antibodies as probes.
Phosphorylation plays a pivotal role in other aspects of NF-κB regulation in addition to DNA binding. These include degradation of IκB proteins, nuclear translocation of NF-κB, transcrip

**DISCUSSION**

Previous studies in our laboratory demonstrated that phosphorylation of Ser^{387} of NF-κB p50 is critical for DNA binding of p50/p50 (19). In this study, we provide evidence that PKAc is the kinase responsible for p50 Ser^{387} phosphorylation in a cAMP-independent manner. Substitution of Ser^{387} abolished p50 phosphorylation by PKAc. Repression of PKAc activity in cells with specific inhibitors blocked p50/p50 DNA binding.

Physical association of PKAc with p50 was found to occur in three different cell types. Moreover, a PKA-specific inhibitory peptide, which binds to the catalytic site of PKAc, blocked the physical association between PKAc and p50. These data reveal that the direct association between PKAc and p50 leads to constitutive phosphorylation of p50 Ser^{387} and subsequent binding of p50/p50 homodimers to DNA.

Phosphorylation plays a pivotal role in other aspects of NF-κB regulation in addition to DNA binding. These include degradation of IκB proteins, nuclear translocation of NF-κB, transcriptional activation, and processing of p105 and p100 to generate p50 and p52, respectively (2, 34). In particular, phosphorylation of p65 has been shown to be required for NF-κB transactivation following induction by cytokines or other stimuli (2). At least four serine residues of p65, including Ser^{276}, Ser^{311}, Ser^{529}, and Ser^{536}, may be phosphorylated by one or more kinases that include PKAc, MSK1, PKCζ, casein kinase II, RSK1, IκB kinases, Ca^{2+}-calmodulin kinase IV, cyclic AMP-dependent kinase, phosphatidylinositol 3-kinase/AKT kinase, and GSK3β (2, 17, 18). Interestingly, the same serine residue in p65 can be targeted by different kinases in different cell types or in response to distinct stimuli. For example, Ser^{276} in the RHD of p65 is phosphorylated by PKAc or MSK1 upon stimulation by lipopolysaccharide or TNFα, respectively (11, 12). Importantly, in resting cells, NF-κB (p50/p65) is associated with IκB and PKAc in the cytoplasm to form a NF-κB-IκB-PKAc complex in which the PKAc activity is blocked by IκB (12). Degradation of IκB in response to lipopolysaccharide and other stimuli leads to activation of PKAc, which in turn phosphorylates Ser^{276} of NF-κB p65 in a cAMP-independent manner. Phosphorylation of Ser^{276} results in a conformational change of p65, which following translocation into the nucleus facilitates recruitment of co-activator CBP/p300 to NF-κB to enhance transcription of target genes (9). Unlike PKAc, MSK1 phosphorylates Ser^{276} of p65 in the nucleus (11).

Based on sequence comparison, Ser^{387} of p65 is the residue directly corresponding to Ser^{387} of p50. However, in contrast to phosphorylation of p65 Ser^{387} by PKAc, we have shown in this study that phosphorylation of p50 Ser^{387} by PKAc is constitutive and does not require degradation of IκB.

This suggests that IκB does not associate with the p50/p50-PKAc complex, which is consistent with the finding that IκB binds efficiently to p50/p50 heterodimers but not to p50/p50 homodimers (35). In accordance with lack of involvement of IκB in the constitutive phosphorylation of p50, our data revealed that TNFα-induced degradation of IκB only led to dramatic nuclear translocation of p50/p50 heterodimers but not p50/p50 homodimers. Therefore, it is unlikely that the same PKAc molecule that phosphorylates p65 in the NF-κB-IκB-PKAc complex is also responsible for constitutive phosphorylation of p50/p50.

Because PKAc is known to function in both the nucleus and cytoplasm (36), it will be important to establish in which cellular compartment p50/p50 homodimers are phosphorylated by the kinase. Although future experiments are required to resolve this question, our in gel kinase experiments suggested that PKAc might phosphorylate p50 in the cytoplasm. If so, it is interesting to consider that p105, the precursor of p50 that is capable of binding to p50 and blocking its nuclear translocation (34), may also serve to inhibit p50 phosphorylation by PKAc. Because in all cells p50 is produced through constitutive processing of p105 (34),
the partial degradation of p105 could relieve the inhibitory effect on p50 phosphorylation by PKAc. It is thus tempting to speculate that constitutive processing of p105 is correlated or even coupled with constitutive phosphorylation of p50.

Sustained activation of NF-κB p50/p65 heterodimers is associated with many chronic inflammatory and autoimmune diseases such as asthma, rheumatoid arthritis, and Alzheimer disease (37–39). This highlights the importance of being able to negatively regulate NF-κB. This inhibitory function can be more effective when p50/p50 homodimers interact with the transcriptional co-repressor histone deacetylase (10). In most resting cells, p50/p50 homodimers are bound to the DNA (10, 23, 24, 27). In particular, constitutive DNA binding of p50/p50 is an apparently important mechanism used by immune cells to suppress expression of NF-κB target genes. For example, p50/p50 homodimers have been found to inhibit interleukin-2 gene expression in resting T lymphocytes (24, 27) and are involved in interleukin-10-mediated anti-inflammation as well as in an autocrine-modulated inhibition of TNFα expression in macrophages (23, 40). It is noted that in a few instances p50/p50 homodimers can also activate gene expression when complexed with co-activators, such as Bcl-3 (29, 41). This cofactor mediated transactivation effect of p50/p50 on one gene would not undermine the suppressive function of the homodimer on other genes. Importantly, our study proposes that regardless of whether p50/p50 functions as a repressor or an activator, the binding of the homodimer to DNA is dependent on phosphorylation of Ser337 by PKAc.

Given the potential complexity for phosphorylation of p50, we cannot rule out that other kinases in addition to PKAc could phosphorylate Ser337. However, our study strongly indicates that in several types of unstimulated cells PKAc serves to constitutively phosphorylate p50 Ser337 directly to enable continuous DNA binding of p50 homodimers. In addition, whereas phosphorylation of p50 is known to be important for DNA binding of NF-κB (p50/65) (21, 22), it remains to be determined how the p50 subunit of NF-κB becomes phosphorylated. One possibility is that the p50 subunit is phosphorylated on Ser337 by PKAc as shown in this study and then partners with the p65 subunit in the NF-κB-p65 complex. Alternatively, the PKAc contained in the NF-κB-p65 complex that serves to phosphorylate p65 (12) could also phosphorylate the p50 subunit component of NF-κB. Future studies are needed to resolve this issue.