Phosphorylation of Serine 337 of NF-κB p50 Is Critical for DNA Binding*

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It has been demonstrated that phosphorylation of the p50 subunit of NF-κB is required for efficient DNA binding, yet the specific phospho-residues of p50 have not been determined. In this study, we substituted all of the serine and conserved threonine residues in the p50 Rel homology domain and identified three serine residues, Ser337, and p50/p50 and p65/p65 homodimer binding to DNA have revealed a conformation often referred to as a “butterfly” (12–16). The DNA recognition loop (L1) in the N-terminal half of NF-κB Rel homology domain mediates base-specific DNA contacts, whereas the C-terminal half is responsible for dimerization and nonspecific DNA contacts (17).

NF-κB activity is regulated by nuclear translocation. In most cell types, p50/p65 heterodimers exist in the cytoplasm as an inactive form associated with the inhibitor protein, IκB. A wide range of stimuli, including cytokines and bacterial and viral products, can induce the phosphorylation of two specific N-terminal serines of IκB-α by the IκB kinase (IKK) complex, which includes IKKα, IKKβ, and IKKγ (NEMO) (18–26). Phosphorylation of IκB-α is targeted for ubiquitination and degradation by the 26 S proteasome (27, 28), enabling NF-κB to rapidly enter the nucleus where it binds to κB sites of target promoters and activates gene transcription. Interestingly, recent studies have shown that NF-κB nuclear localization is regulated by reversible acetylation of its p65 subunit. Acetylation of p65 by CBP/p300 prevents binding to nuclear IκB-α, whereas deacetylation of p65 by HDAC promotes efficient binding to nuclear IκB-α and allows an IκB-α-dependent nuclear export of the NF-κB complex (29).

At the gene promoter level, the ability of NF-κB to bind DNA and to activate gene transcription is controlled by phosphorylation. Phosphorylation of the p65 subunit regulates the transcriptional activity of NF-κB. Specifically, when serine 276 (Ser276) in the Rel homology domain is phosphorylated by the PKA catalytic subunit, p65 is able to associate with coactivator CBP/p300, whereas unphosphorylated p65 associates with co-repressor histone deacetylase (10, 30, 31). In addition, Ser320 and Ser326 in the p65 transactivation domain are phosphorylated by casein kinase II and IKKα, respectively, which increase NF-κB transactivation potential (30–34).

On the other hand, phosphorylation of the p50 subunit regulates the DNA binding of NF-κB. The NF-κB p50 subunit is known to be phosphorylated on serine residues, and much less significantly on threonine residues, but not on tyrosine residues (35, 36). Previous studies have demonstrated the biological significance of p50 phosphorylation on NF-κB DNA binding. In tumorigenic adenovirus type 12 transformed cells, NF-κB is translocated into the nucleus but fails to bind to DNA, mainly because of the hypophosphorylated state of the p50 subunit (37). Upon cytokine treatment (tumor necrosis factor-α and interleukin-1β) of Ad12-transformed cells, p50 becomes phosphorylated, allowing NF-κB to bind to DNA (38). It has also been reported that p50 phosphorylation induced by phorbol

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1 The abbreviations used are: IKK, IκB kinase; PKA, protein kinase A; PKAc, catalytic subunit of protein kinase A; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; CBP, CREB (cAMP-response element-binding protein)-binding protein; GST, glutathione S-transferase.
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EXPERIMENTAL PROCEDURES

Cell Lines—Monolayer cultures of COS-7 cells (American Type Culture Collection) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen), 10% fetal bovine serum, 2 mm l-glutamine, 100 mm units/ml penicillin, 0.1 mg/ml streptomycin (Invitrogen). Whole Cell Extract Preparation—Cells from a 10-cm dish were washed twice with 5 ml of cold phosphate-buffered saline, scraped off with a rubber policeman in 0.9 ml of cold 250 mM Tris-HCl (pH 8.0), and pelleted by microcentrifugation at 6000 rpm for 5 min at 4°C. The pelleted cells were resuspended in 100 μl of cold 250 mM Tris-HCl (pH 8.0) and lysed by “freeze-thawing” four times with dry ice and ethanol and 37°C water. Lysates were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant fraction (whole cell extract) was stored as aliquots at −80°C.

Plasmids—pGEX-mp50 plasmid was constructed by subcloning cDNA sequence encoding residues 1–363 of mouse p50 protein into pGEX-4T-2, which was used as the template for further mutagenesis to produce 16 mutant p50 proteins. pCMV-hp50 (40) contains a cDNA sequence encoding residues 1–399 of human p50 flanked by T7 and Sp6 promoters and was employed as the template for mutagenesis for mutant p50 plasmids used in COS-7 cell transfection.

Mutagenesis—Site-directed mutagenesis was performed with either a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions or by a PCR method. Briefly, to mutate p50 sequences in pCMV-hp50 by PCR, two complementary oligonucleotides harboring the desired mutations were used in combination with T7 and Sp6 primers to amplify the full-length p50 sequence with the desired mutations. The products from the second PCR round were subcloned into pCMV plasmids.

Electrophoretic Mobility Shift Assay (EMSA)—A double-stranded oligonucleotide probe (5′-tagAGGCTGGGATTCCCATG-3′) of the MHC class I R1 enhancer site, which contains a recognition sequence for NF-κB, was labeled using [γ-32P]ATP at 167 μCi/ml (25 mm HEPES, pH 7.5, 10 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, 1 mM CaCl2, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 20 μM ATP). GST-p50 fusion proteins were purified by glutathione-agarose beads, washed extensively with NTNZ (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.1% NP-40, 10 mM ZnCl2), and resolved on a SDS-PAGE. The gel was dried and analyzed by autoradiography. Alternatively, the in vitro kinase assay was performed without [γ-32P]ATP, and the p50 proteins were tested for their DNA binding ability in EMSA with 32P-labeled R1 oligonucleotides.

RESULTS

Ser365, Ser337, and Ser242 of p50 Are Critical for DNA Binding—Members of the Rel protein family are known to be phosphorylated. For example, in response to stimuli, the Drosophila NF-κB homologue, dorsal, is phosphorylated in the cytoplasm on serine residues Ser70, Ser33, Ser103, and Ser213 (19),推测 the signal transduction activities are regulated by phosphorylation on Ser327, Ser329, and Ser336 (10, 30–34), whereas the phosphodesires on p50 have yet to be identified.

To determine the potential phosphosites that are critical for p50 DNA binding in vivo, we tested the DNA binding abilities of wild type and point mutant p50 proteins in a mammalian cellular environment. All 16 of the serine residues in the human p50 protein Rel homology domain were substituted with alanine. Wild type and mutant p50 plasmids were transfected into COS-7 cells, which have very low background expression of NF-κB proteins. After 48 h, whole cell extracts of COS-7 cells were prepared, and the overexpressed exogenous p50 proteins were examined for their abilities to bind to an R1 DNA oligonucleotide. As shown in Fig. 1A, untransfected COS-7 cells exhibited negligible NF-κB DNA binding (lane 1), as expected. Of all the mutant p50 proteins, only three, S65A, S337A, and S342A, displayed dramatically reduced DNA binding abilities as compared with the wild type p50 (compare lanes 4, 17, and 18 with lane 2). All of the other serine to alanine mutations had no significant effect on DNA binding. In addition, we also selected the most highly conserved threonine residues in p50 based on the homology among p50, p65, and Dorsal proteins and replaced them with alanine to test their effects on p50 DNA binding. As shown in Fig. 1, p50 mutants T158A, T228A, T263A, and T341A were still capable of binding to DNA (compare lanes 19-22 with lane 2). Western blotting with anti-p50 antibody was performed to confirm the presence of equal amounts of exogenous p50 proteins (Fig. 1B).

It was confirmed in vitro that residues Ser365, Ser337, and
mutant p50 S65A, S337A, and S342A were co-expressed with NF-κB.

The crystal structure represented here has been described previously (15, 16). One p50 subunit is shown in green, and the other in blue. The DNA is in red. Ser65, Ser337, and Ser342 of one p50 subunit are indicated. The residue numbers here refer to human p50, which in mouse p50 are Ser63, Ser335, and Ser341, respectively.

Ser342 are critical for p50 DNA binding. Bacterially produced wild type and mutant p50 proteins were kinased by wheat germ extract. Whereas the DNA binding activities of wild type and several other p50 mutant proteins were strongly increased when kinased, the DNA binding activities of the p50 mutants S65A, S337A, and S342A were unaffected (data not shown).

Dimerization of p50 Is Not Affected by Mutating Ser65, Ser337, and Ser342 to Alanine. Wild type (WT) and mutant p50 S65A, S337A, and S342A were co-expressed with NF-κB p65 and labeled with 35S by in vitro transcription/translation followed by immunoprecipitation with anti-p65 antibody. The immunoprecipitated p65 and its associated p50 were washed, resolved by SDS-PAGE, and visualized by autoradiography (lanes 5–8). To confirm the equal expression, p50 proteins were also expressed alone, labeled with 35S by in vitro transcription/translation, and then immunoprecipitated with anti-p50 antibody. The immunoprecipitated p50 were washed and visualized by autoradiography (lanes 1–4). The positions of p65 and p50 are indicated.

Ser342 of p50 is phosphorylated in vivo. Nuclear extracts from HeLa cells (40 μg) were immunoprecipitated (IP) with goat anti-human p50 antibody (Santa Cruz), resolved by SDS-PAGE, and analyzed by Western blot probed with anti-RX-phosphoserine/phosphothreonine antibody or rabbit anti-human p50 antibody.

Required for DNA binding. The p50 mutants S65A, S337A, and S342A were coexpressed, respectively, with p65 and labeled with 32P by in vitro transcription and translation followed by immunoprecipitation with anti-p65 antibody. The immunoprecipitates were then resolved by SDS-PAGE and visualized by autoradiography. As shown in Fig. 3, wild type and all three mutant p50 proteins (S65A, S337A, and S342A) could be co-immunoprecipitated with p65, with the same apparent efficiency (lanes 5–8). The equal expression of wild type and mutant p50 proteins was confirmed by expressing p50 alone followed by immunoprecipitation with anti-p50 antibody (Fig. 3, lanes 1–4). These results clearly demonstrate that the inability of these three mutant p50 proteins to bind DNA is not merely because of a failure to dimerize.

Ser337 of p50 Is a Phosphoresidue Critical for DNA Binding—We next wished to inquire whether phosphorylation of residues 65, 337, and 342 are important for p50 DNA binding. First, Ser65, Ser337, and Ser342 were mutated to aspartic acid (Asp), which contains a negative charge and can sometimes mimic phosphorylated serines. As shown in Fig. 4A, the p50 mutant proteins S65D, S337D, and S342D failed to bind DNA (lanes 3–5). We then investigated whether replacing these residues with threonine, a phospho-accepting amino acid that can generally substitute for serine, could restore the DNA binding. As shown in Fig. 4A, p50 mutant proteins S65T and S337T failed to bind DNA (lanes 2 and 7 with lane 2), suggesting that the potential phosphorylation of Ser65 and Ser337 is essential for efficient DNA binding. Interestingly, threonine could not replace serine at residue 342, as the DNA binding ability of mutant p50 S342T was seriously impaired (Fig. 4A, lane 8). This suggested either that Ser342 is not phosphorylated or that a serine at residue 342 is specifi-
Fig. 6. Phosphorylation of Ser337 by PKA in vitro elevates DNA binding of p50. A, purified PKA catalytic subunit (5 units) were incubated with 4 μg of GST and GST-p50 wild type (WT) or mutant 249–363 peptides in an in vitro kinase assay with [γ-32P]ATP. The proteins were fractionated by SDS-PAGE and visualized by autoradiography. The positions of GST and GST-p50 fusion proteins are indicated. B, full-length wild type or mutant GST-p50 proteins (30 ng) were incubated with or without purified PKA catalytic subunit and then tested for DNA binding by EMSA. The position of p50/p50 homodimer bound to DNA is indicated.

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Phosphorylation is crucially required for the phosphorylation at this position. Western blotting confirmed the expression of mutant exogenous p50 proteins (Fig. 4B).

Even though the above results identified three important serine residues (Ser65, Ser337, and Ser342), we consider that Ser337 is most likely a phosphoresidue that is critical for DNA binding. The sequence surrounding Ser337, LRRKSDLE, is a consensus substrate site for many kinases, including PKA, and Ser337 can be substituted with threonine, a potential alternate phospho-accepting species. Moreover, the position of Ser337 is fully exposed, providing easy access for a kinase. On the other hand, Ser65 and Ser342 are not part of any known kinase consensus sequences. Further, Ser65 is likely to be sensitive to mutation because of its position on the DNA recognition loop of p50 (Fig. 2). The ability of threonine to substitute for Ser337 may simply be because this conservative mutation is not disruptive to the structure of the p50 DNA recognition loop (see “Discussion”). Thus, although we cannot definitively rule out the possibility that Ser65 and Ser342 are phosphoresidues important for DNA binding, we focused on Ser337 to further investigate the effect of its potential phosphorylation on DNA binding.

We examined whether Ser337 is indeed phosphorylated in vivo. The immediate sequence surrounding Ser337 in both mouse and human p50 is RRRKSDLE, which can be detected by a PKA substrate antibody that specifically recognizes phosphorylated serine or threonine residues in the sequence Arg-Xaa-PSer/Thr (RX(pS/T)). To test the phosphorylation state of Ser337 in a cellular environment, p50 proteins were immunoprecipitated from nuclear extracts of HeLa cells with anti-p50 antibody and analyzed by Western blotting with either the PKA substrate antibody (anti-RX(pS/T)) or anti-p50 antibody. As shown in Fig. 5, Western blotting with the PKA substrate antibody revealed a 50-kDa protein (lane 1). The identity of this protein as authentic p50 was confirmed by anti-p50 Western blotting (lane 2). This finding provides evidence that p50 Ser337 can be phosphorylated in vivo.

To confirm the ability of PKA to specifically phosphorylate residue Ser337 of p50, the purified PKA catalytic subunit (Sigma) was incubated with bacterially purified wild type or mutant p50 249–363 polypeptides in an in vitro kinase assay. As shown in Fig. 6A, the PKA catalytic subunit (PKAc) could phosphorylate wild type p50 but not GST control protein (compare lanes 1 and 2). As expected, PKAc failed to phosphorylate both p50 S337A mutant and S337A/S342A double mutant, which lack the PKA site (Fig. 6A, lanes 3 and 5). Notably, the mutation of Ser342 to alanine alone was unable to disrupt phosphorylation on Ser337 by PKA (Fig. 6A, lane 4). These results prove definitively that Ser337 of p50 is a PKA kinase site and can be phosphorylated by PKA in vitro.

We next tested whether phosphorylation of Ser337 is critical for DNA binding. We incubated both wild type and mutant S337A full-length p50 proteins with PKA catalytic subunit in the in vitro kinase assay and examined how the phosphorylation of residue Ser337 affects p50 DNA binding. As shown in Fig. 6B, phosphorylation of Ser337 by PKA dramatically increased the ability of wild type p50 to bind DNA (compare lane 2 with lane 1). By contrast, p50 mutant S337A, which is unable to be phosphorylated by PKA, failed to bind DNA following PKA treatment (compare lane 4 with lane 3).

**DISCUSSION**

In this study, we have identified three serine residues, Ser65, Ser337, and Ser342, that are critical for p50 DNA binding. A disruption of p50 dimerization ability is not the cause of the loss of DNA binding that results from individually mutating each of these serine residues. In particular, we have proved both in vitro and in vivo that p50 DNA binding is regulated by phosphorylation of serine 337. Our studies highlight how the function of NF-κB can be distinctively regulated by the specific phosphorylation of each of its subunits. Whereas phosphorylation of Ser337 on p50 regulates DNA binding by the p50 subunit, phosphorylation of Ser276, Ser529, and Ser536 on p65 regulates NF-κB transactivation (30–34).

Recently, Ser276 of p65 was shown to be phosphorylated by the PKA catalytic subunit present in the cytosolic NF-κB-IκB-PKAc complex upon degradation of IκB. The phosphorylation of Ser276 of p65 induces a conformational change that enhances the binding of CBP/p300 by weakening the interaction between the Rel homology domain and the C-terminal transactivation domain (10, 30, 31). Interestingly, Ser337 in the Rel homology domain of p50 is the residue directly corresponding to Ser276 of p65, the only regulatory phosphoresidue in the Rel homology domain of p65. Specifically, the sequence surrounding p50 Ser337 (LRRKSDLE), which is the only serine residue of p50 fitting a consensus substrate site for PKA, is highly similar to that of p65 Ser276 (LRRPSDE). Intriguingly, our preliminary findings also suggest that Ser337 of p50 and Ser276 of p65 may play similar and critical roles in DNA binding of the respective subunits, which raises the question of whether these two residues are co-regulated by a common enzyme. Although we have shown that Ser337 is a target for phosphorylation by PKA in vitro, making it highly probable that p50 is regulated by PKA,
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it should be noted that the sequence surrounding Ser337 also fits the profile of several other kinases such as CaMII, casein kinase II, and protein kinase G. Nonetheless, the conformational change induced by the phosphorylation of p50 Ser337 could be different from that induced by phosphorylation of p65 Ser276 because p50 lacks a transactivation domain. The crystal structures of DNA-bound NF-κB show that Ser337 is proximal to the hinge region linking the N-terminal and C-terminal domains of p50 (46). Serine 337 resides on the outer surface of p50, making it easily accessible by kinases. Phosphorylation at this position is critical, as substitution of Ser337 with threonine fully retains p50 DNA binding, whereas merely substituting a negative charge at this position with aspartic acid disables p50 DNA binding. We speculate that phosphorylation of Ser337 enables DNA binding of p50 by inducing a conformational change in the hinge region of p50.

Although it is evident that phosphorylation of Ser337 of p50 is critical for DNA binding, it is unclear why Ser276 and Ser342, the other two residues identified in this study, are important for DNA binding. Notably, neither of these two residues has a consensus site for a known kinase. In the case of Ser276, the crystal structures of the NF-κB DNA complex reveal that this residue is located on the DNA recognition loop of p50, which projects into the major groove of the DNA (Fig. 2). Although Ser276 itself does not make specific contacts with DNA, it is required to maintain the secondary structure of the DNA recognition loop by forming potentially crucial van der Waals contacts with residues Gly375 and Phe376 using the hydroxyl side chain. These contacts presumably become disrupted by the substitution of Ser276 with alanine, causing destabilization of the loop structure and loss of p50 DNA binding. By contrast, mutation of Ser342 to threonine preserves the van der Waals contacts with residues Gly375 and Phe376, thus maintaining the DNA recognition loop and the DNA binding ability of p50. The reason that mutation of Ser276 to aspartic acid failed to bind DNA is likely because it creates an additional hydrogen bond with Arg298, the residue that forms a specific DNA contact with a guanine. Taken together, our mutational results correlate perfectly with the predictions based on structural information, suggesting that Ser276 is sensitive to mutation because of its importance in maintaining the p50 DNA recognition loop rather than being potentially regulated by phosphorylation. In the case of Ser342, it is unclear why this residue is important for p50 DNA binding. Phosphorylation may not be involved, as substitution with threonine failed to retain p50 DNA binding, although it is known that some kinases, notably IKKs, strongly prefer serine over threonine (46). In addition, despite their proximity, Ser342 is not involved in phosphorylation of Ser337, as phosphorylation of Ser337 by PKA was not disrupted by mutation of Ser342. In summary, it is intriguing to consider that Ser276 and Ser342 are important for DNA binding for reasons other than phosphorylation and that DNA binding of p50 is regulated solely by phosphorylation of Ser337. Future studies will identify the actual cellular p50 Ser337 kinase and the mechanism by which this phosphorylation affects NF-κB DNA binding.

Control of DNA binding by phosphorylation of p50 Ser337 represents a novel mechanism, which adds to the complexity of NF-κB regulation that includes nuclear translocation and transactivation of p65. This finding has major biological implications, as p50 functions in the DNA binding of both the transactivator NF-κB p50/p65 heterodimer and transrepressor p50/p50 homodimer and, therefore, has the potential to both positively and negatively modulate the transcription of NF-κB-controlled genes. It will be important to determine how the discovery of this new NF-κB mechanism contributes to understanding the development, physiology, and pathology of certain diseases.

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