Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65

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NF-κB represents a family of eukaryotic transcription factors participating in the regulation of various cellular genes involved in the immediate early processes of immune, acute-phase, and inflammatory responses. Cellular localization and consequently the transcriptional activity of NF-κB is tightly regulated by its partner IκBα. Here, we show that the p65 subunit of NF-κB is acetylated by both p300 and PCAF on lysines 122 and 123. Both HDAC2 and HDAC3 interact with p65, although only HDAC3 was able to deacetylate p65. Acetylation of p65 reduces its ability to bind κB-DNA. Finally, acetylation of p65 facilitated its removal from DNA and consequently its IκB-mediated export from the nucleus. We propose that acetylation of p65 plays a key role in IκBα-mediated attenuation of NF-κB transcriptional activity which is an important process that restores the latent state in post-induced cells.

The NF-κB/Rel family of inducible transcription factors is involved in the expression of numerous genes involved in processes such as growth, development, apoptosis, and inflammatory and immune responses (1, 2). The Rel family includes p65 (RelA), p105/p50, p100/p52, RelB, and c-Rel, which homo- or heterodimerize to form transcriptionally competent or repressive complexes referred to as NF-κB (3). The most abundant form of NF-κB is a p50/p65 heterodimer in which p65 contains the transcriptional activation domain. The activity of NF-κB is regulated by its subcellular localization. In unstimulated cells, NF-κB exists in an inactive form sequestered in the cytoplasm by its inhibitor, IκB. The IκB family includes several members of which the best characterized is IκBα (2). Cell activation by a multitude of extracellular signals (4) converges on phosphorylation of IκB by IκB kinase, which triggers its rapid ubiquitination and degradation by the proteasome (5). Degradation of IκB unmasks the nuclear localization signal (NLS)7 present in NF-κB, which then enters the nucleus to activate target gene expression.

A key step to controlling NF-κB activity is the regulation of NF-κB subcellular localization through its interaction with IκB in both pre-induced and post-induced cellular states. IκB contains both a nuclear import sequence (6, 7), and a strong nuclear export sequence that utilizes the exportin/CRM1 pathway (8–12). One of the target genes of NF-κB is IκBα, resulting in rapid induction of newly synthesized IκBα protein, which enters the nucleus and dissociates NF-κB from κB-DNA to repress NF-κB function (13, 14). NF-κB/IκB complexes are exported to the cytoplasm where they may serve for additional rounds of activation or restore the original latent state (6, 8).

The activity of NF-κB is regulated by transcriptional coactivators that may function by bridging sequence-specific activators to the basal transcriptional machinery and also play a role in chromatin remodeling via their intrinsic histone acetyltransferase (HAT) or deacetylase (HDAC) activity (15). p65 binds to CBP (CREB-binding protein) and its homologue p300 as well as PCAF (p300/CBP-associated factor), whereas p50 fails to recruit transcriptional coactivators (16–21). p65 phosphorylation by protein kinase A stimulates NF-κB-dependent gene expression by enhancing its interaction with CBP (21). Enhancement of NF-κB transcriptional activity requires the acetyltransferase activity of CBP/p300 (20) and PCAF (19).

The p65 subunit of NF-κB was recently shown to be acetylated (22). It was proposed by Chen et al. that reversible acetylation regulates the interaction between p65 and IκBα and, therefore, controls the duration of the NF-κB response. Here, we show that p65 is acetylated on dual lysine residues K122/123 by p300 and PCAF and deacetylated by HDAC3. Contrary to Chen et al., we could not demonstrate any significant effect of acetylation on the interaction between p65 and IκBα. Rather, we show that acetylation reduces binding of p65 to κB-containing DNA, facilitating its removal by IκBα and subsequent export to the cytoplasm. We propose that acetylation of p65 contributes to the mechanism of post-induction turn-off of NF-κB-mediated transcription.

7 The abbreviations used are: NLS, nuclear localization signal; HAT, histone acetyltransferase; HDAC, histone deacetylase; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; FBS, fetal bovine serum; ATP, dithiothreitol; TSA, trichostatin A; EMSA, electrophoretic mobility shift assay; HIV-1, human immunodeficiency virus, type 1; ChIP, chromosomal immunoprecipitation; IL-8, interleukin-8; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; LMB, leptomycin B; CMV, cytomegalovirus; FAT, factor acetyltransferase; LTR, long terminal repeat; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TK, thymidine kinase.
**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions and Antibodies Used—**Eukaryotic expression vectors for T7-{	extit{La}}b, LTR-h-rw, PCAF, p300, HDAC1, HDAC2, HDAC3, and HDAC5 have been described previously (23–25). FLAG-p65 (wt) was generated by PCR using the oligonucleotides (the FLAG sequence is highlighted in boldface): (forward) 5'-TCCAGGCTT-CACCATGACATCAAAAGCATGAGCAACGAGGATCTT-CCCTCTACCTTCCCG-3' (reverse) 5'-CGGGATCTCCCTTGATAGGCTGATCTGACTCAG-3'. PCR fragments were cloned into pTarget (Promega) and the clones were fully sequenced. FLAG-p65 was used to generate p65 mutants. Mutagenesis was performed by QuickChange site-directed mutagenesis (Stratagene). Mutations were generated using the following pairs of mutagenic oligonucleotide primers (mutations are highlighted in boldface): K122A/K123A: (forward) 5'-CGGGATCTCCCTTGATAGGCTGATCTGACTCAG-3' and (reverse) 5'-CTCTGCTCCAGGTCCCGACACACTGGATTCCCAG-3'. K122R/K123R: (forward) 5'-CGGGATCTCCCTTGATGTCGAGGGC-GGACCTTGAGCAGG-3' and 5'-CTCTGCTCCAGGTCCCGACACACTGGATTCCCAG-3'.

**Cell Culture and Immunological Techniques—**Jurkat cells were cultured in RPMI 1640 Glutamax medium (Invitrogen) supplemented with 10% heat-inactivated FBS, penicillin and streptomycin. HeLa and 293 cells were propagated in Dulbecco’s modified Eagle’s medium with 10% FBS. Transfections were performed using calcium phosphate or, where indicated, by LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Amounts of DNA are as indicated in the figure legends. The total amount of expression vectors was kept constant by using empty-vector DNA. Where indicated, cells were treated with LMB (10 mM) overnight and during labeling.

For preparation of cytoplasmic and nuclear extracts, cells were washed twice in cold phosphate-buffered saline, resuspended in 400 μl of 10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, in the absence or presence of 200 μM TSA (Sigma), for 1 h at 37 °C. Reactions were analyzed by SDS-PAGE. Gels were fixed in 30% methanol and 10% acetic acid, enhanced, dried and exposed to x-ray film at ~70 °C.

**EMSA—**EMSA was performed using 10^6 cpm of [γ-32P]ATP-labeled HIV-1 X box probe. Reactions were performed in binding buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 0.01% Nonidet P-40, 1 mM ZnSO4, 1 mM DTT) for 15 min at room temperature, resolved on 4% acrylamide gels, dried, and exposed to x-ray film. Chromatin Immunoprecipitation Assay—Six 60-mm-diameter Petri dishes of transfected 293 cells were used per chromatin immunoprecipitation reaction, performed essentially as described previously (27). To cleared chromatin extracts, 2 μg of FLAG M2 monoclonal antibody was added. PCR was performed in the presence of 0.11 μCi of [α-32P]dCTP. PCR products were analyzed by electrophoresis on 4% TBE polyacrylamide gels and autoradiography. The IL-8 promoter intensities obtained from immunoprecipitates were first normalized to the starting chromatin extracts (input). The -fold enrichment is defined as the ratio of the normalized intensities for the transfected samples to the mock-transfected sample. PCR primer sets for the human IL-8 promoter region −121/−61 and the human IL-8 upstream region −1042/−828 have been described previously (28).

**RESULTS**

p65 Is Acetylated on Lysines 122 and 123 by p300 and PCAF—p300/CBP and PCAF bind to the p65 but not the p50 subunit of NF-κB and regulate NF-κB transcriptional activity. To test whether p65 is modified by acetylation, we performed in vivo acetylation assays. Jurkat cells were untreated or treated with PMA to activate NF-κB, or the HDAC inhibitor TSA or a combination of both, biosynthetically labeled for 1 h with [3H]sodium acetate (NaAc) or [35S]methionine plus cysteine (Cys/Met), and lysed, and protein was immunoprecipitated using either p65-specific or p50-specific antisera. Acetylated p65 was observed only in PMA-treated cells (Fig. 1A). TSA treatment alone did not result in p65 acetylation. However, treatment with both PMA and TSA enhanced p65 acetylation when compared with PMA treatment alone. In contrast, no acetylation of p50 was observed (lanes 5–8). This is consistent with a recent report showing that p50 acetylation can be detected only in the presence of HIV-1 Tat (29). This experiment shows that p65 is acetylated in vivo, and the acetylation is dependent on NF-κB activation.

Because the acetylationtransferase activities of p300/CBP and PCAF have been shown to be important for transcriptional activation of p65 (19, 20), we investigated whether p65 may be a substrate for these transcriptional coactivators. p65 was acetylated by both p300 and PCAF in vitro (Fig. 1B). No acetylation of GST- or GST-p50 was observed (data not shown). Peptide mapping analysis showed that lysines 122 and 123 within peptide 3 (p3) are the only residues acetylated by p300 and PCAF in vitro (Fig. 1C). Thus, GST-p65 wt or a mutant in which Lys-122 and Lys-123 were mutated to alanine (KK-AA) were used as substrates in in vitro acetylation assays. Substitution of the two lysines completely abrogated acetylation of p65 by both p300 and PCAF (Fig. 1D). No acetylation of GST-p50 was observed (lanes 3 and 6). Coomassie Blue staining confirmed that equivalent amounts of proteins were loaded (data not shown). To confirm that K122/L23 were also acetylated in vitro, 293 cells were transfected with vectors expressing either FLAG-p65 wild type or mutants where the two acetylated lysines were changed to arginines (KK-RR) or alanines (KK-AA). Cells were pulse-labeled with either [3H]NaAc or [35S]Met/Cys, lysed, and protein was immunoprecipitated using anti-FLAG antibody (Fig. 1E). In vitro acetylation was observed in cells transfected with wild type p65 but not with KK-RR or KK-AA mutants. Metabolic labeling using [35S]methionine plus cysteine confirmed that p65 wild type and mutant proteins were expressed to equivalent levels in transfected cells. These results show that p65 is acetylated at dual lysine residues, Lys-122 and Lys-123, by both p300 and PCAF.
Simultaneous treatment of cells with PMA and TSA enhanced the acetylation of p65 in vivo (Fig. 1A), implying a tight regulation of p65 acetylation by deacetylases in vivo. We first determined the HDACs that interact with p65 in vivo. Both HDAC2 and HDAC3 interacted with p65, whereas no interaction was observed between HDAC1 and p65 (Fig. 2A). The presence of HDAC1, HDAC2, and HDAC3 in the immunoprecipitates is shown (Fig. 2A, lanes 3–8). It was recently reported that p65 interacts directly with HDAC1 but not HDACs 2 or 3 (30). The discrepancy between the results obtained by Ashburner et al. (30) and those shown in Fig. 2A may be due to the different antibodies used.

![Image](72x237 to 550x737)

**Fig. 1.** **p65 is acetylated at lysines 122 and 123 in vivo and in vitro.** **A**, p65 acetylation in vivo requires NF-κB activation. Jurkat cells were mock-treated (lanes 1 and 5), treated overnight with PMA 10 ng/ml (lanes 2 and 6), TSA 200 nM (lanes 3 and 7), or PMA plus TSA (lanes 4 and 8). p65 and p50 were immunoprecipitated from cells that had been pulse-labeled with either [3H]NaAc or [35S]Met/Cys. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Autoradiographs corresponding to [3H]p65 and [3H]p50 were exposed to film for 5 days and 30 days, respectively. **B**, p65 is acetylated in vitro by both p300 and PCAF. Histone H3, 100 ng (lanes 1–8) or varying amounts of GST-p65 as indicated were incubated in acetylation buffer with either recombinant p300 (lanes 1–4) or recombinant PCAF (lanes 5–8). Reaction products were separated by 4–20% SDS-PAGE, and the gels were Coomassie Blue-stained (bottom panel), dried, and visualized by autoradiography (top panel). **C and D**, p300 and PCAF acetylate lysines 122 and 123 in vitro. **C**, synthetic peptides (1 μg) corresponding to amino acids 21–60 (p1), 61–99 (p2), 111–130 (p3), 210–230 (p4), and 290–320 (p5) of p65 were incubated with PCAF and [14C]acetyl-CoA for 1 h at 37 °C. Reaction products were resolved in 16.5% Tris-Tricine acrylamide gels followed by autoradiography. Shown is the quantification of the radiolabeled peptides. **D**, GST-p65 wild type, GST-p65KK-AA (AA), and GST-p50 were incubated in acetylation buffer with either recombinant p300 (lanes 1–3) or recombinant PCAF (lanes 4–6). Reaction products were analyzed by SDS-PAGE and autoradiography. **E**, lysines 122 and 123 of p65 are acetyl-acceptors in vivo. FLAG-tagged p65 wild type, KK-RR, or KK-AA were immunoprecipitated from transfected 293 cell extracts that had been pulse-labeled with either [3H]NaAc or [35S]Met/Cys. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.
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We next analyzed the ability of HDAC2 and HDAC3 to deacetylate p65 in vitro. FLAG-p65, immunopurified from transfected 293 cells that had been labeled with [3H]NaAc, was incubated with HDAC2 or HDAC3 in the presence or absence of TSA. HDAC3 but not HDAC2 was able to deacetylate p65 in vitro (Fig. 2B). Deacetylation of p65 by HDAC3 was inhibited by TSA. Western blot analysis showed that comparable amounts of p65 were present in all samples. Both HDAC2 and HDAC3 deacetylated histone H3 in vitro (lanes 7–9) showing that the purified HDACs were active. To establish the role of HDAC3 in p65 deacetylation in vivo, FLAG-p65 was immunopurified from transfected cells that had been labeled with [3H]NaAc or [35S]Met/Cys. Acetylation of p65 was enhanced in cells cotransfected with p65 and PCAF or p300 (Fig. 2C) confirming that p65 is a substrate for p300 and PCAF in vivo. In contrast, cotransfection of HDAC3 with p65 significantly reduced p65 acetylation. TSA treatment inhibited HDAC3-mediated deacetylation of p65 (lane 5). No effect of HDAC2 on p65 acetylation was observed (lanes 6 and 7). [35S]Met/Cys labeling shows the level of expression of the different plasmids used. Taken together, the results show that deacetylation of p65 is regulated in vivo by p300, PCAF, and HDAC3.

Acetylation of p65 Does Not Affect Its Interaction with IκBα—It was recently reported that acetylation of p65 prevents its interaction with IκBα (22). We therefore examined whether mutation of acetyl-acceptor lysines 122 and 123 of p65 affected its interaction with IκBα. As shown in Fig. 3A, the affinities for IκBα of p65 wild type, KK-RR, and KK-AA were equivalent. We next examined directly whether or not the acetylated form of p65 interacted with IκBα (Fig. 3B). FLAG-p65 and T7-IκBα were either transfected alone or cotransfected under conditions in which IκBα was overexpressed relative to p65. Extracts of transfected cells that had been pulse-labeled with either [3H]NaAc or [35S]Met/Cys were subjected to immunoprecipitation using anti-FLAG (lane 1) or anti-IκBα (lane 2). The extracts from IκBα-p65-cotransfected cells were subjected to sequential rounds of immunoprecipitation with anti-T7 (lanes 3 and 4) followed by a third round with anti-FLAG (lane 5). Most of [35S]p65 was found in association with IκBα, because immunodepletion of T7-IκBα resulted in immunodepletion of p65 from the cells (compare lane 3 to lane 5). Analysis of acetylated p65 showed that all of [3H]p65 was also detected in complexes
Acetyl transferase PCAF affects its DNA binding activity. A, immunopurified FLAG-p65 and PCAF were incubated in acetylation buffer in the presence or absence of [1-14C]acetyl-CoA. [1-14C]-labeled p65 protein was analyzed by SDS-PAGE and autoradiography. B, varying amounts of acetylated FLAG-p65 (p65Ac, lanes 1–3) and non-acetylated FLAG-p65 (p65, lanes 4–6) were incubated with [32P]labeled χB probe and analyzed by EMSA. Reactions were resolved by 4% acrylamide gel electrophoresis and analyzed by autoradiography. C, various amounts of nuclear extracts (NE) of HeLa cells transfected with either FLAG-p65 (wt), FLAG-p65KK-RR (RR), or FLAG-p65KR-RA (AA) were analyzed by EMSA as described in b. D, expression of p65 wild type (wt), KK-RR (RR), and KK-AA (AA) in nuclear extracts of transfected HeLa cells detected by Western blotting using anti-FLAG.

Acetylation of p65 Reduces Its Binding to χB-DNA—The crystal structure of the p50/p65 heterodimeric bound to DNA revealed that, among the p65 residues involved in DNA binding, acetyl-acceptor lysines 122 and 123 identified in this report are the only residues that contact the DNA in the minor groove (31). Because acetylation would be expected to neutralize the positive charge on Lys-122 and Lys-123 ε-amino groups, we sought to determine how acetylation of p65 might affect its interaction with χB-DNA. To investigate this, FLAG-p65 immunopurified from transfected 293 cells (Fig. 4A, Coomassie Blue staining) was acetylated or mock-acetylated in vitro by PCAF (Fig. 4A, autoradiography). EMSA analysis of the acetylated and mock-acetylated products showed that acetylation of FLAG-p65 reduces its ability to bind χB-DNA (Fig. 4B). Similar results were observed when p300-acetylated p65 or PCAF-acetylated GST-p65 were used (data not shown). Nuclear extracts of HeLa cells transfected with p65 wild type, KK-RR, and KK-AA were next analyzed for binding to χB-DNA (Fig. 4C). After normalization for the expression level of the transfected vectors (Fig. 4D), wild type p65 and KK-RR mutant bound χB-DNA with similar affinities while the KK-AA mutant was incompetent in binding DNA (Fig. 4C). Thus, consistent with predictions from crystal structure analysis (31), the acetyl-acceptor lyses 122 and 123 identified in this report participate in high affinity binding of p65 to χB-DNA.

p65 Is Acetylated in the Nucleus and Accumulates in the Cytoplasm—Because acetylation plays a role in destabilizing the p65/χB-DNA interaction, we next investigated whether p65 acetylation occurs in the cytoplasm or nucleus. HeLa cells transfected with FLAG-p65 expression vector were untreated or treated with leptomycin B (LMB), which blocks the exportin/CRM1 pathway and inhibits IκBα-mediated export of NF-κB resulting in the accumulation of p65 in the nucleus (9–11). Cellular protein synthesis was blocked 24 h post-transfection by incubation in Met/Cys-deficient media. Cells were maintained in the presence of LMB throughout the experiment. Immediately prior to lysis, cells were pulse-labeled with [3H]NaAc. FLAG-p65, immunoprecipitated separately from cytoplasmic and nuclear extracts, was analyzed by Western blot (Fig. 5A, lanes 1–4). In the absence of LMB, p65 was found in both cytoplasmic and nuclear compartments (Fig. 5A, lanes 1–2). LMB treatment led to accumulation of p65 in the nucleus (Fig. 5A, lanes 3–4). Acetylated p65 in immunoprecipitates was analyzed by SDS-PAGE and autoradiography (Fig. 5A, lanes 5–8). In the absence of LMB, acetylated p65 accumulated in the cytoplasm (Fig. 5A, compare lane 1 to 2 and lane 5 to 6). In contrast, LMB treatment resulted in localization of acetylated p65 in the nucleus (Fig. 5A, compare lane 3 to 4 and lane 7 to 8). These results suggest that p65 is acetylated in the nucleus and accumulates in the cytoplasm in the absence of LMB.

To further examine the site of p65 acetylation in cells, p65 was blocked in the cytoplasm by its inhibitor, IκBα. IκBα causes retention of p65 in the cytoplasm by masking the NF-κB NLS (2). The experiment was performed under conditions where protein synthesis was inhibited as described for Fig. 5A. In the absence of IκBα, p65 was found in both cytoplasmic and nuclear fractions, whereas in the presence of IκBα, p65 localized exclusively to the cytoplasmic fraction (Fig. 5B). Autoradiography analysis showed that, in the absence of IκBα, acetylated p65 was detected in both cytoplasmic and nuclear fractions at levels approximately corresponding to the amount of p65 detected in each compartment (Fig. 5B, lane 5 and 7). However, when p65 was blocked in the cytoplasm by coexpression with IκBα, no acetylated p65 was detected (Fig. 5B, lanes 6 and 8). This experiment shows that cytoplasmic p65 is not a substrate for acetylation. Taken together, these results demonstrate that p65 is acetylated in the nucleus and accumulates in the cytoplasm.

To further characterize the effect of IκBα on p65 acetylation in vivo, we performed the same experiment as in Fig. 5B except under conditions in which protein synthesis was not inhibited. Under these conditions, NF-κB and IκBα shuttle between the cytoplasm and nucleus. [3H]S-p65 was found in both cytoplasmic and nuclear extracts of p65-transfected cells (Fig. 5C, lanes 1 and 3). When p65 and IκBα were cotransfected, [3H]S-p65 was found mainly in the cytoplasm but was also present in the nucleus, due to expression of newly synthesized p65 (Fig. 5C, lanes 2 and 4). Analysis of acetylated p65 ([3H]S-p65) showed that in the absence of IκBα, [3H]S-p65 was found in both cytoplasmic and nuclear fractions (Fig. 5C, compare lane 5 to 7). However, when p65 and IκBα were coexpressed under conditions of ongoing protein synthesis, acetylated p65 accumulated in the cytoplasm (Fig. 5C, compare lane 6 to 8). Interestingly, IκBα was found to enhance p65 acetylation under these conditions (Fig. 5C, compare lanes 5 to 6). Thus, acetylation of p65 may facilitate its nuclear export by IκBα.

Immunofluorescence analysis was performed to analyze the subcellular localization of acetylation-competent (wild type) and acetylation-incompetent (KK-RR or KK-AA) forms of p65. HeLa cells were transfected with either FLAG-p65 wild type, KK-RR, or KK-AA expression vectors, and cells were stained with anti-FLAG to detect p65 and anti-IκBα antibodies to detect the induction of endogenous IκBα. Wild type p65 was found in the nucleus only in cells in which IκBα was not induced (Fig.
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5D, panels 1 and 2). Induction of endogenous IκBα by p65 led to its export to the cytoplasm (data not shown). Cotransfection of p65 and IκBα led to the export of p65 to the cytoplasm in the majority of transfected cells (Fig. 5D, panels 5 and 6). The p65KK-AA mutant was localized almost exclusively in the nucleus (Fig. 5D, panels 7 and 8). This is likely due to its weak transcriptional activity (Fig. 6A) and, consequently, lack of IκBα induction by p65KK-AA (data not shown). In agreement with this, cotransfection of p65KK-AA and IκBα expression vectors lead to accumulation of p65KK-AA in the cytoplasm (Fig. 5D, panels 9 and 10). The p65KK-RR mutant induced high level expression of endogenous IκBα (data not shown), likely due to its high transcriptional activity (Fig. 6A). However, in a significant number of cells, this mutant localized in the nucleus even in the presence of strong induction of IκBα expression (Fig. 5D, panels 11 and 12, see cell indicated by the arrow) suggesting that it binds tightly to DNA, which prevents its cytoplasmic export by IκBα.

Acetylation of p65 Represses Its Transcriptional Activity and Is Involved in the Attenuation of p65-mediated Transcription—It has been shown previously that p300/CBP and PCAF are transcriptional coactivators for NF-κB (16-21). Recruitment of these coactivators by p65 may induce localized chromatin remodeling via their intrinsic histone-directed acetylation activities resulting in enhancement of p65-mediated transcription. To investigate the role of factor acetyltransferase (FAT) activity of p300 and PCAF toward p65 on its transcriptional activity in vivo, HeLa cells were transfected with plasmids expressing wild type p65 or mutants in which the two acetyl-acceptor residues (Lys-122 and Lys-123) were substituted with arginines (KK-RR) or alanines (KK-AA). The KK-RR mutation, which conserves the positive charge, enhanced p65 transcriptional activity, whereas alanine substitutions that neutralize the positive charge reduced p65-mediated transcription of the HIV-1 LTR-luciferase reporter (Fig. 6A). Because the KK-RR mutant and wild type p65 bound to κB-DNA equivalently after normalization to the expression level of the transfected vectors (Fig. 3C), the enhanced transcriptional activity of p65KK-RR is not due to increased DNA binding. These results suggest that acetylation of p65 down-regulates its transcriptional activity. Furthermore, when fused to the GAL4 DNA-binding domain, the transcriptional activities of wild type p65, KK-RR, and KK-AA mutants were equivalent toward 5XGAL4-luciferase reporter plasmid (Fig. 6B).

We next analyzed the combined effects HAT and FAT activities of p300 and PCAF, HDAC3, and their respective enzymatic activity mutants on p65-mediated transcriptional activation of HIV-1 LTR-luciferase reporter (Fig. 6C). As previously reported, p300 and PCAF enhanced p65 transcriptional activity. Interestingly, p300 and PCAF coactivated p65KK-RR transcriptional activity to a significantly higher level than p65 wild type. Thus, elimination of acetyl-acceptors for FAT activity leads to higher coactivation between p65 and HATs. On the other hand, HDAC3 repressed p65KK-RR transcriptional activity more than that of p65 wild type. These results, taken together with our other experiments, strongly suggest that HAT activity of p300 and PCAF potentiates p65-mediated transcription, whereas their FAT activity is repressive.

The opposing effects of HAT and FAT activities of p300 and PCAF in NF-κB-dependent gene expression led us to the hypothesis that the FAT activity of p300 and PCAF may be involved in turning off NF-κB-dependent gene expression. NF-κB-dependent gene expression is in part regulated by induction of its inhibitor, IκBα. IκBα binds to and dissociates NF-κB from κB-DNA thus contributing to attenuation of NF-κB transcriptional activity (6, 8). The data presented suggest a model in which acetylation of p65 may contribute to IκBα-mediated export of p65 to the cytoplasm resulting in accumulation of acetylated p65 in the cytoplasm. To examine this hypothesis, we
analyzed the effect of IκBα on the transcriptional activity of wild type p65 and KK-RR mutant. IκBα was more efficient in inhibiting wild type p65-mediated transcriptional activity than...
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In this report, we examined the functions of p300 and PCAF coactivators in NF-κB-mediated transcription. Both p300 and PCAF acetylate p65 and, moreover, target the same residues within p65: lysines 122 and 123 that are important for high affinity binding of p65 to κB-DNA. We identified HDAC3 as the histone deacetylase responsible for p65 deacetylation. Fig. 8 shows a schematic representation of the proposed role of p65 acetylation in NF-κB-mediated transcription. Following immune stimulation of cells, which results in degradation of IκBα, NF-κB is translocated to the nucleus via the newly exposed NLS in p65 and binds tightly to κB-DNA elements. Recruitment of p300 and PCAF to the promoter region results in activation of NF-κB-mediated transcription presumably through their associated histone-directed acetylase activity, which induces localized chromatin remodeling. Subsequently, acetylation of p65 by p300 or PCAF at two DNA-binding residues, lysines 122 and 123, lowers its affinity for κB-DNA. This facilitates removal of NF-κB from enhancer elements by newly synthesized IκBα whose expression is induced by NF-κB, and subsequent export of NF-κB-IκBα from the nucleus to the cytoplasm. The NF-κB-IκBα cytoplasmic complex can subsequently either establish a post-induced latent state or serve for additional rounds of activation following deacetylation of p65 by HDAC3. Thus, acetylation of p65 is essential for turning off NF-κB-mediated gene expression.

Recently, the reversible acetylation of p65 was reported by Chen et al. (22). Although both studies show that reversible acetylation of p65 by p300 and HDAC3 plays a critical role in NF-κB-transcriptional activity, the function attributed to p65 acetylation contrasts sharply between the two studies. Chen et al. proposed a model in which deacetylation of p65 promotes binding between NF-κB and IκBα, which mediates export of the complex to the cytoplasm to establish the latent state. In contrast, we have shown that acetylation of p65 reduces its interaction with κB DNA and thereby promotes IκBα-mediated nuclear export. We were unable to demonstrate any significant effect of p65 acetylation on its interaction with IκBα (Fig. 3). Because Chen et al. did not identify the specific acetyl acceptor residues present in p65, their conclusions were based on experiments using the broadly acting HDAC inhibitor, TSA, to enhance acetylation. However, TSA induces global effects on acetylation in the cell and would influence the function of many acetylated substrates, which include coactivators, components of the basal transcription machinery, and proteins involved in nuclear import (34, 35). Chen et al. showed that cotreatment of cells with tumor necrosis factor α and TSA enhances p65 DNA-binding activity. In contrast, a direct comparison of κB-DNA binding by acetylated and non-acetylated forms of p65 showed that acetylation of p65 lowered its affinity for κB-DNA (Fig. 4), which consequently promotes its IκBα-mediated export to the cytoplasm (Figs. 5, 7, and 8). Using a GST-IκBα pull-down assay, Chen et al. showed that cotransfection of p300 and p65 reduces the ability of GST-IκBα to interact with p65 and concluded that acetylation of p65 prevents its interaction with IκBα. We directly analyzed binding of IκBα to acetylated p65 by communoprecipitation analysis using extracts from either [3H]NaAc or [35S]Met/Cys-labeled cells and found that most of the acetylated p65 in cells can be found in association with IκBα. Indeed, given that acetylation of p65 occurs in the nucleus (Fig. 5), the finding that acetylated p65 accumulates in the cytoplasm in an IκBα-dependent manner indicates that the interaction between p65 and IκBα is not abolished, because studies using knock-out mice have shown that nuclear export of NF-κB depends on its interaction with IκBα. The discrepancy between the conclusions reached by the two studies may be due to differences in the experimental approaches used.

DISCUSSION

In this report, we examined the functions of p300 and PCAF coactivators in NF-κB-mediated transcription. Both p300 and PCAF acetylate p65 and, moreover, target the same residues within p65: lysines 122 and 123 that are important for high affinity binding of p65 to κB-DNA. We identified HDAC3 as the histone deacetylase responsible for p65 deacetylation. Fig. 8 shows a schematic representation of the proposed role of p65 acetylation in NF-κB-mediated transcription. Following immune stimulation of cells, which results in degradation of IκBα, NF-κB is translocated to the nucleus via the newly exposed NLS in p65 and binds tightly to κB-DNA elements. Recruitment of p300 and PCAF to the promoter region results in activation of NF-κB-mediated transcription presumably through their associated histone-directed acetylase activity, which induces localized chromatin remodeling. Subsequently, acetylation of p65 by p300 or PCAF at two DNA-binding residues, lysines 122 and 123, lowers its affinity for κB-DNA. This facilitates removal of NF-κB from enhancer elements by newly synthesized IκBα whose expression is induced by NF-κB, and subsequent export of NF-κB-IκBα from the nucleus to the cytoplasm. The NF-κB-IκBα cytoplasmic complex can subsequently either establish a post-induced latent state or serve for additional rounds of activation following deacetylation of p65 by HDAC3. Thus, acetylation of p65 is essential for turning off NF-κB-mediated gene expression.

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**Fig. 8.** Schematic model for the proposed role of p300- and PCAF-induced acetylation of p65 in regulating NF-κB-mediated transcription. See text for details.
The importance of histone-mediated NF-kB export from the nucleus and consequently the termination of NF-kB-dependent transcription has been provided from studies using I-kBα knockout mice (36, 37). However, the mechanism by which I-kBα removes NF-kB from DNA is unknown. The p50/p65 heterodimer has a particularly high affinity for k-B-DNA, between 10^{-10} and 10^{-14} (38, 39), whereas the affinity of I-kBα for the p50/p65 heterodimer is 10^{-9} (40). Thus, the NF-kB/DNA interaction would need to be destabilized so that I-kBα can compete for the removal of NF-kB from DNA. In agreement with this, Munshi et al. (41) found that I-kBα was not able to remove NF-kB from DNA in the context of the INF-β enhancer. The crystal structure of the p50/p65 heterodimer bound to k-B-DNA revealed that, among p65 residues involved in DNA binding, lysines 122 and 123 are the only residues that heterodimer has a particularly high affinity for p300 and PCAF, which, together, are histone acetyltransferases that their acetylation activity would need to be regulated in a sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic transcriptional coactivators that function by bridging the sequence-specific activators to the basal transcriptional machinery and play a role in chromatin remodeling via their intrinsic.