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Post-activation Turn-off of NF- κ B-dependent Transcription Is Regulated by Acetylation of p65*

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NF- κ B 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- κ B is tightly regulated by its partner I κ B α . Here, we show that the p65 subunit of NF- κ B 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 κ B-DNA. Finally, acetylation of p65 facilitated its removal from DNA and consequently its I κ B α -mediated export from the nucleus. We propose that acetylation of p65 plays a key role in I κ B α -mediated attenuation of NF- κ B transcriptional activity which is an important process that restores the latent state in post-induced cells.

The NF- κ B/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- κ B (3). The most abundant form of NF- κ B is a p50/p65 heterodimer in which p65 contains the transcriptional activation domain. The activity of NF- κ B is regulated by its subcellular localization. In unstimulated cells, NF- κ B exists in an inactive form sequestered in the cytoplasm by its inhibitor, I κ B. The I κ B family includes several members of which the best characterized is I κ B α (2). Cell activation by a multitude of extracellular signals (4) converges on phosphorylation of I κ B by I κ B kinase, which triggers its rapid ubiquitination and degradation by the proteasome (5). Degradation of I κ B

unmasks the nuclear localization signal (NLS)¹ present in NF- κ B, which then enters the nucleus to activate target gene expression.

A key step to controlling NF- κ B activity is the regulation of NF- κ B subcellular localization through its interaction with I κ B in both pre-induced and post-induced cellular states. I κ B α 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- κ B is I κ B α , resulting in rapid induction of newly synthesized I κ B α protein, which enters the nucleus and dissociates NF- κ B from κ B-DNA to repress NF- κ B function (13, 14). NF- κ B-I κ B 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- κ B 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- κ B-dependent gene expression by enhancing its interaction with CBP (21). Enhancement of NF- κ B transcriptional activity requires the acetyltransferase activity of CBP/p300 (20) and PCAF (19).

The p65 subunit of NF- κ B was recently shown to be acetylated (22). It was proposed by Chen *et al.* that reversible acetylation regulates the interaction between p65 and I κ B α and, therefore, controls the duration of the NF- κ B 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 I κ B α . Rather, we show that acetylation reduces binding of p65 to κ B-containing DNA, facilitating its removal by I κ B α and subsequent export to the cytoplasm. We propose that acetylation of p65 contributes to the mechanism of post-induction turn-off of NF- κ B-mediated transcription.

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¹ 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; DTT, 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-I κ B α , LTRluc-wt, PCAF, p300, HDAC1, HDAC2, HDAC3, and HDAC3mut 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'-TCCAAGCTT-CACCATGGACTACAAAGACGATGACGACAAGGACGAACTGTT-CCCCCTCATCTTCCCG-3' (reverse) 5'-CGCGGATCCCCCTTAGGAGCTGATCTGACTCAG-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 QuikChange site-directed mutagenesis (Stratagene). Mutations were generated with the following pairs of mutagenic oligonucleotide primers (mutations are highlighted in boldface): K122A/K123A: (forward) 5'-CTGGGAATCCA-GTGTGTGGCGGCGCGGACCTGGAGCAGG-3' and (reverse) 5'-CCTGCTCCAGGTCCCGCGCCGACACACTGGATTCCAG-3'. K122R/K123R: (forward) 5'-CTGGGAATCCAAGTGTGTGGAGGCGG-GGACCTGGAGCAGG-3' (reverse) and 5'-CCTGCTCCAGGTCCCGC-CTCCTCACACACTGGATTCCAG-3'. Mutated clones were fully resequenced. The mutated plasmids were designated as p65KK-AA and p65KK-RR. To produce GST fusion proteins, p65 WT and p65KK-AA were cloned into pGEX-4T (Amersham Biosciences). GST-p65 and GST-p65KK-AA fusion proteins were expressed and purified as previously described (26). Antibodies used were anti-p65 (C-20), anti-p50 (E-10), anti-I κ B α (FL), anti-HDAC1 (H-11), and anti-HDAC2 (C-19) (Santa Cruz Biotechnology), anti-FLAG M2 (Sigma), and anti-HDAC3 (23).

Cell Culture and Immunological Techniques—Jurkat cells were cultured in RPMI 1640 GlutamaxI medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 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 nM) overnight and during labeling.

For preparation of cytoplasmic and nuclear extracts, cells were washed twice in cold phosphate-buffered saline, resuspended in 400 μ l of 10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors, and incubated on ice for 20 min. Nonidet P-40 was added to the cells to a final concentration of 0.5% followed by mixing and centrifugation for 30 s at 4 °C. Supernatants (corresponding to cytoplasmic extracts) were collected, and nuclear extracts were prepared by resuspending pellets in 50 μ l of 50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and protease inhibitors. Proteins were extracted by agitation for 20 min at 4 °C and clarified by centrifugation. Total cell extracts were prepared by lysing the cells in Triton buffer (300 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton, and protease inhibitors). Immunoprecipitation and Western blotting were performed as described previously (24).

For immunofluorescence, HeLa cells were transfected with FLAG-tagged p65 wild type, p65KK-RR or p65KK-AA. Cells were fixed 24 h after transfection in a solution containing 4% paraformaldehyde, 0.5% Triton, and 5% FBS. Cells were double-stained with anti-FLAG monoclonal antibody and rabbit polyclonal anti-I κ B α followed by incubation with Cy5-conjugated anti-mouse and anti-rabbit fluorescein isothiocyanate for detection of FLAG-p65 and I κ B α , respectively.

Acetylation and Deacetylation Assays—*In vivo* acetylation and deacetylation assays were performed as follows. Transfected HeLa or 293 cells 24 h post-transfection were washed three times in phosphate-buffered saline and incubated in media minus methionine/cysteine supplemented with 2% FBS for 2 h at 37 °C. Cell cultures were halved and incubated in media containing either [³⁵S]Met plus Cys at 1 mCi/ml or [³H]NaAc at 1 mCi/ml for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and resuspended in lysis buffer (300 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton X-100, and protease inhibitors).

In vitro assays for protein acetylation were performed using peptides or GST-p65 fusion protein as described previously (24). For *in vitro* deacetylation assays, 293 cells were transfected with FLAG-p65, FLAG-HDAC2, or FLAG-HDAC3 expression plasmids. 24 h after transfection, FLAG-p65-transfected cells were labeled with [³H]NaAc for 1 h at 37 °C. Extracts were prepared and subjected to immunoprecipitation using anti-FLAG antibody. FLAG-p65 was eluted using FLAG peptide overnight at 4 °C. FLAG-HDAC2 and FLAG-HDAC3 were kept on the beads. The purified FLAG-p65 was incubated with FLAG-HDAC2 or FLAG-HDAC3 in HDAC buffer (10 mM Tris, pH 8, 10 mM NaCl, 10% glycerol), in the absence or presence of 200 mM TSA (Sigma), for 1 h at

37 °C. Reactions were analyzed by 10% 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 10⁴ cpm of [³²P]ATP-labeled HIV-1 κ B probe. Reactions were performed in binding buffer (25 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 0.01% Nonidet P-40, 1 μ M 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 μ g of FLAG M2 monoclonal antibody was added. PCR was performed in the presence of 0.11 μ Ci of [³²P]dCTP. PCR products were analyzed by electrophoresis on 4% 1 \times 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/-826 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- κ B and regulate NF- κ B 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- κ B, 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- κ B activation.

Because the acetyltransferase 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 acetylation by 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 K122/123 were also acetylated *in vivo*, 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 vivo* 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.

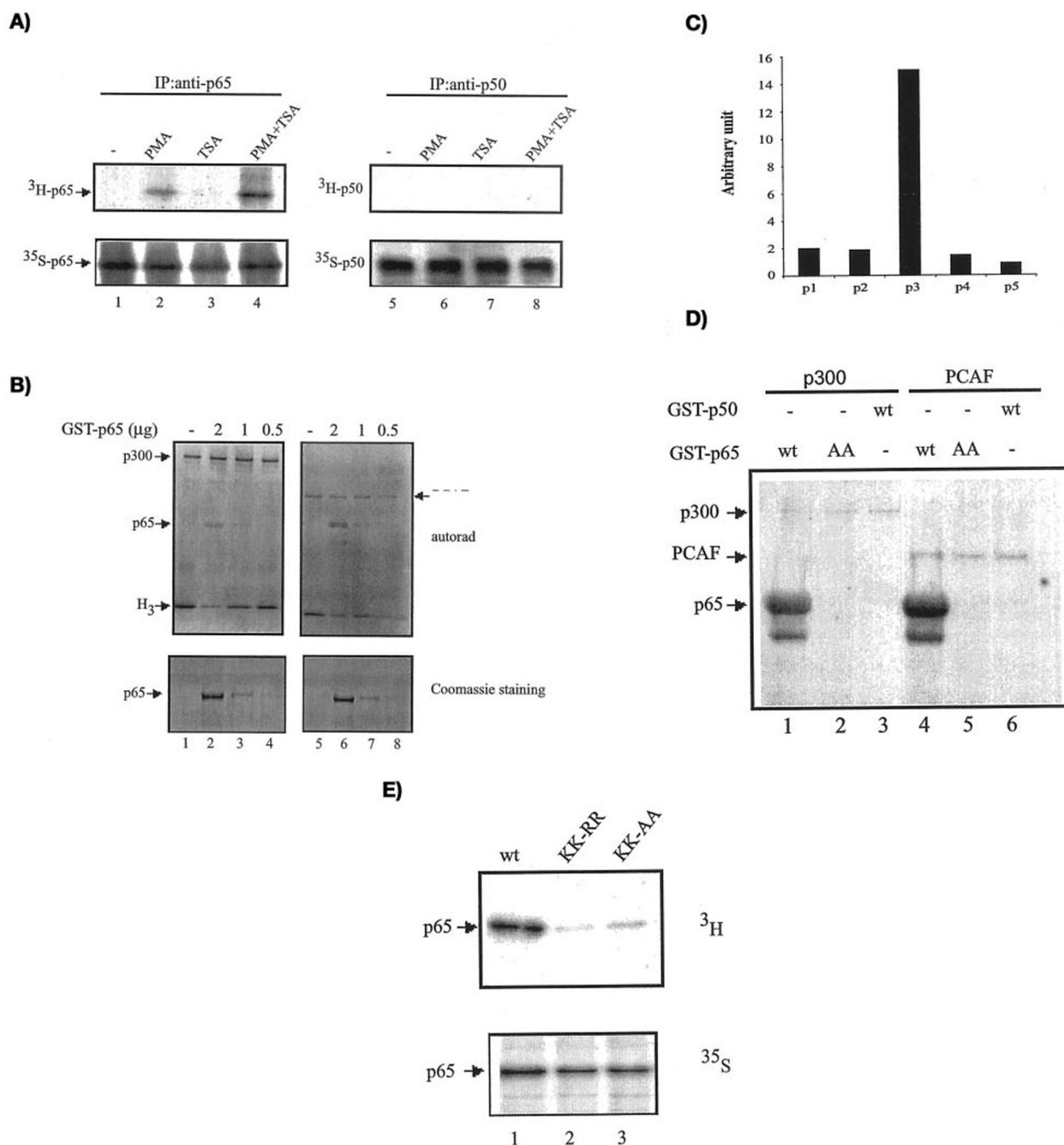


FIG. 1. p65 is acetylated at lysines 122 and 123 *in vivo* and *in vitro*. *A*, p65 acetylation *in vivo* requires NF- κ B activation. Jurkat cells were mock-treated (lanes 1 and 5), treated overnight with PMA 10 ng/ml (lanes 2 and 6), TSA 200 nM (lanes 3 and 7), or PMA plus TSA (lanes 4 and 8). p65 and p50 were immunoprecipitated from cells that had been pulse-labeled with either [^3H]NaAc or [^{35}S]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 1–4) 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 [^{14}C]acetyl-CoA for 1 h at 37 $^{\circ}\text{C}$. 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 [^{35}S]Met/Cys. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

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 7–9). 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.

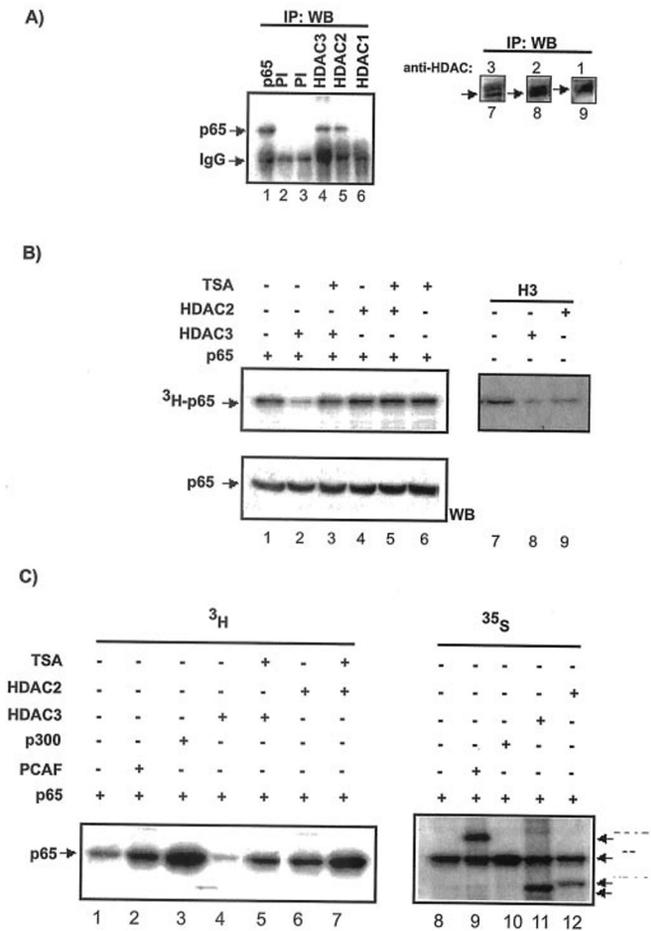


FIG. 2. HDAC3 deacetylates p65 *in vitro* and *in vivo*. A, p65 interacts with HDAC2 and HDAC3 but not HDAC1. HeLa extracts were immunoprecipitated using anti-p65, anti-HDAC1, anti-HDAC2, and anti-HDAC3 antibodies. Two different pre-immune sera (PI) were used as controls. The presence of p65 in immunoprecipitates was analyzed by Western blotting using anti-p65 antibody. The presence of HDAC3, HDAC2, and HDAC1 in the respective immunoprecipitates is shown (right panel, lanes 7–9). The arrows indicate the HDAC proteins. The upper band in lane 7 corresponds to IgG. B, HDAC3 but not HDAC2 deacetylates p65 *in vitro*. HDAC2, HDAC3, and ³H-labeled FLAG-p65 ([³H]p65) were immunopurified separately from 293 cells transfected with the respective expression plasmids. [³H]p65 was incubated in deacetylation buffer alone (lane 1) or with HDAC2 or HDAC3 in the presence or absence of TSA (lanes 2–6). Acetylated histone H3 was incubated in deacetylation buffer (lanes 7–9) either alone or in the presence of HDAC2 or HDAC3 as a control. Reactions were analyzed by SDS-PAGE and autoradiography (top panels) or Western blotting using anti-FLAG antibody (lower panel). C, HDAC3 deacetylates p65 *in vivo*. HeLa cells transfected with FLAG-p65 expression vector either alone or with the indicated plasmids were metabolically labeled with [³H]NaAc in the presence or absence of TSA (lanes 1–7) or [³⁵S]Met/Cys (lanes 8–12). FLAG-p65 was immunoprecipitated from cell extracts, and bound complexes were resolved by SDS-PAGE and detected by autoradiography.

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 immunoprecipitated from transfected cells that had been labeled with

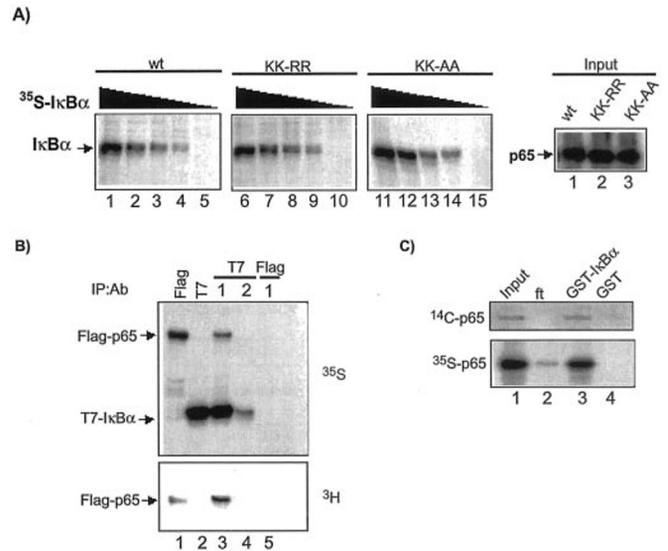


FIG. 3. Acetylation of p65 does not affect its interaction with IκBα. A, immunoprecipitated FLAG-p65 wt, KK-RR, and KK-AA were incubated with 2-fold decreasing amounts of ³⁵S-labeled IκBα. The complexes were washed extensively and retention of IκBα was analyzed by SDS-PAGE and autoradiography. p65 wt and mutants were also analyzed directly by Western blotting (input, lanes 1 to 3). B, extracts of HeLa cells transfected with FLAG-p65 (lane 2) or T7-IκBα (lane 5) alone were immunoprecipitated with the indicated antibodies. Extracts of HeLa cells, cotransfected with FLAG-p65 and T7-IκBα, were subjected to two sequential immunoprecipitations using anti-T7 antibody overnight at 4 °C (lanes 3–4) followed by a third immunoprecipitation using anti-FLAG antibody overnight at 4 °C (lane 5). Immunoprecipitates were washed extensively and analyzed by 10% SDS-PAGE and autoradiography. C, *in vitro* acetylated p65 (top panel) or *in vitro* translated ³⁵S-labeled p65 (lower panel) was incubated with GST-IκBα or GST for 2 h at 4 °C. The beads were washed five times in buffer containing 250 mM KCl and resuspended in loading buffer. The presence of p65 was analyzed by SDS-PAGE followed by autoradiography. Flow-through (ft) corresponding to GST-IκBα is shown in lane 2.

[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 acetylation 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-T7 (lane 2). The extracts from IκBα-p65-cotransfected cells were subjected to two 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

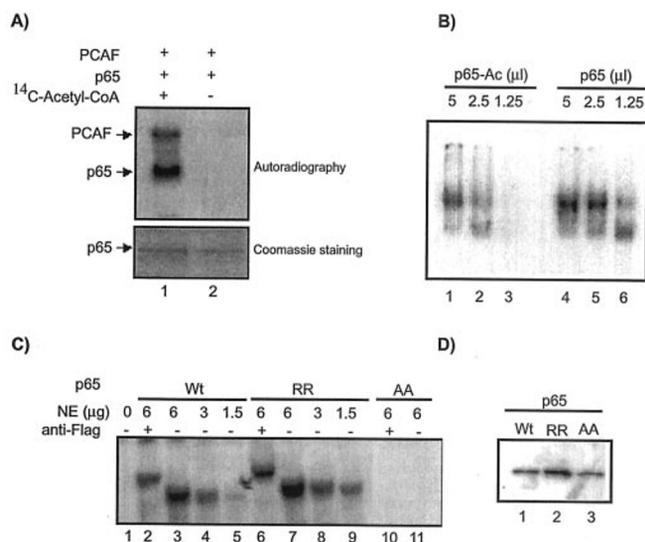


FIG. 4. p65 acetylation by PCAF affects its DNA binding activity. *A*, immunopurified FLAG-p65 and PCAF were incubated in acetylation buffer in the presence or absence of [^{14}C]acetyl-CoA. ^{14}C -labeled p65 protein was analyzed by SDS-PAGE and autoradiography. *B*, varying amounts of acetylated FLAG-p65 (p65-Ac, lanes 1–3) and non-acetylated FLAG-p65 (p65, lanes 4–6) were incubated with ^{32}P -labeled κB 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-p65KK-AA (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.

with $\text{I}\kappa\text{B}\alpha$ (compare lanes 3 to 5, lower panel). Moreover, using GST- $\text{I}\kappa\text{B}\alpha$ and *in vitro* acetylated p65 (^{14}C -p65) or *in vitro* translated ^{35}S -labeled p65 (^{35}S -p65) we show that GST- $\text{I}\kappa\text{B}\alpha$ is able to interact equally with acetylated and non-acetylated p65 (Fig. 3C, compare lanes 3 to 1). Taken together, the data in Fig. 3 strongly suggests that acetylation of p65 does not abolish its binding to $\text{I}\kappa\text{B}\alpha$.

Acetylation of p65 Reduces Its Binding to κB -DNA—The crystal structure of the p50/p65 heterodimer 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 κB -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 κB -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 κB -DNA (Fig. 4C). After normalization for the expression level of the transfected vectors (Fig. 4D), wild type p65 and KK-RR mutant bound κB -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 lysines 122 and 123 identified in this report participate in high affinity binding of p65 to κB -DNA.

p65 Is Acetylated in the Nucleus and Accumulates in the Cytoplasm—Because acetylation plays a role in destabilizing the p65/ κB -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 $\text{I}\kappa\text{B}\alpha$ -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, $\text{I}\kappa\text{B}\alpha$. $\text{I}\kappa\text{B}\alpha$ 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 $\text{I}\kappa\text{B}\alpha$, p65 was found in both cytoplasmic and nuclear fractions, whereas in the presence of $\text{I}\kappa\text{B}\alpha$, p65 localized exclusively to the cytoplasmic fraction (Fig. 5B). Autoradiography analysis showed that, in the absence of $\text{I}\kappa\text{B}\alpha$, 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 $\text{I}\kappa\text{B}\alpha$, 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 $\text{I}\kappa\text{B}\alpha$ 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 $\text{I}\kappa\text{B}\alpha$ shuttle between the cytoplasm and nucleus. ^{35}S -p65 was found in both cytoplasmic and nuclear extracts of p65-transfected cells (Fig. 5C, lanes 1 and 3). When p65 and $\text{I}\kappa\text{B}\alpha$ were cotransfected, ^{35}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 p65) showed that in the absence of $\text{I}\kappa\text{B}\alpha$, [^3H]p65 was found in both cytoplasmic and nuclear fractions (Fig. 5C, compare lane 5 to 7). However, when p65 and $\text{I}\kappa\text{B}\alpha$ were coexpressed under conditions of ongoing protein synthesis, acetylated p65 accumulated in the cytoplasm (Fig. 5C, compare lane 6 to 8). Interestingly, $\text{I}\kappa\text{B}\alpha$ 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 $\text{I}\kappa\text{B}\alpha$.

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- $\text{I}\kappa\text{B}\alpha$ antibodies to detect the induction of endogenous $\text{I}\kappa\text{B}\alpha$. Wild type p65 was found in the nucleus only in cells in which $\text{I}\kappa\text{B}\alpha$ was not induced (Fig.

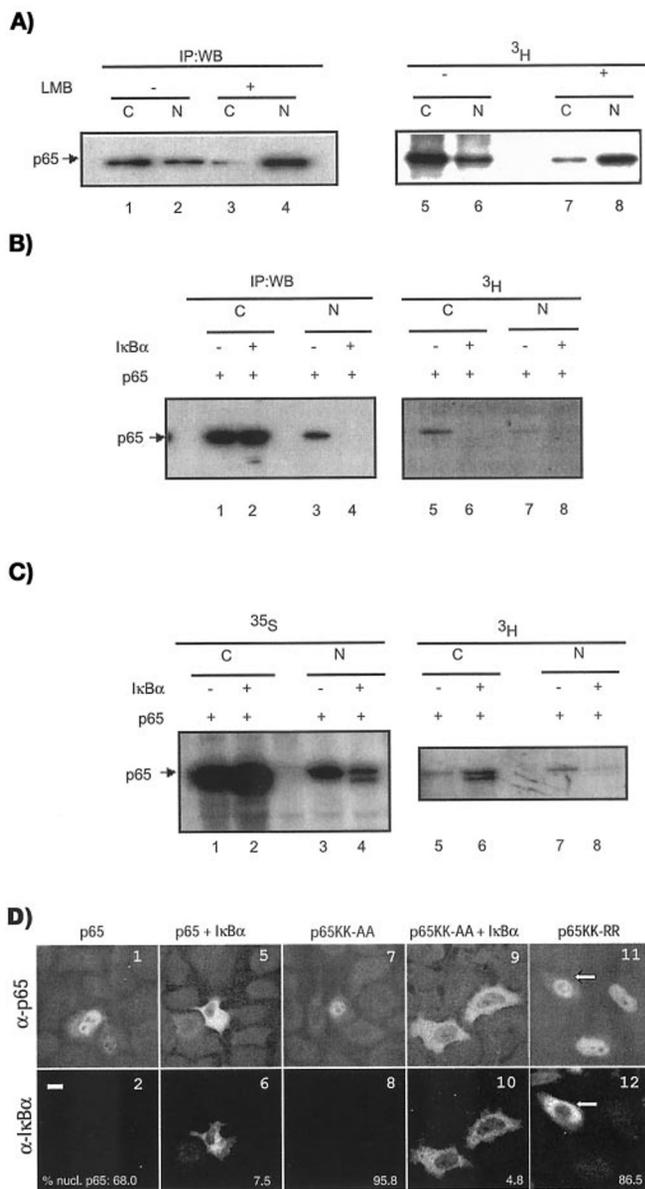


FIG. 5. p65 is acetylated in the nucleus and accumulates in the cytoplasm. *A*, HeLa cells transfected with FLAG-p65 were mock-treated or treated with LMB overnight as indicated. Cells were incubated in Met/Cys-deficient media for 3 h at 37 °C and labeled in the same media supplemented with [³H]NaAc for 1 h at 37 °C. p65 was immunoprecipitated from cytoplasmic (C) and nuclear (N) extracts. Immunoprecipitates were resolved by SDS-PAGE, and the presence of p65 was analyzed by Western blot (lanes 1–4) and autoradiography (lanes 5–8). *B*, the experiment was performed as described in *A* except that HeLa cells were transfected with FLAG-p65 expression vector alone or cotransfected with IκBα expression vector as indicated. *C*, acetylation of p65 facilitates its nuclear export by IκBα. The experiment was performed as described in *B* except that protein synthesis in the transfected cells was not inhibited. Cells were labeled with either [³⁵S]Met/Cys or [³H]NaAc as indicated. *D*, mutation of acetyl-acceptor lysines affects induction of IκBα expression and its subsequent IκBα-mediated export from the nucleus. HeLa cells transfected with the indicated expression plasmids were stained with Cy5-conjugated anti-mouse and anti-rabbit fluorescein isothiocyanate for detection of FLAG-p65 and IκBα, respectively. The arrow highlights a p65KK-RR-expressing cell in which IκBα is induced. Average percentages of cells displaying nuclear localization of p65 are shown in the lower right corner of each panel. Results are derived from examination of at least 200 transfected cells from two independent experiments. Bar represents 25 μm.

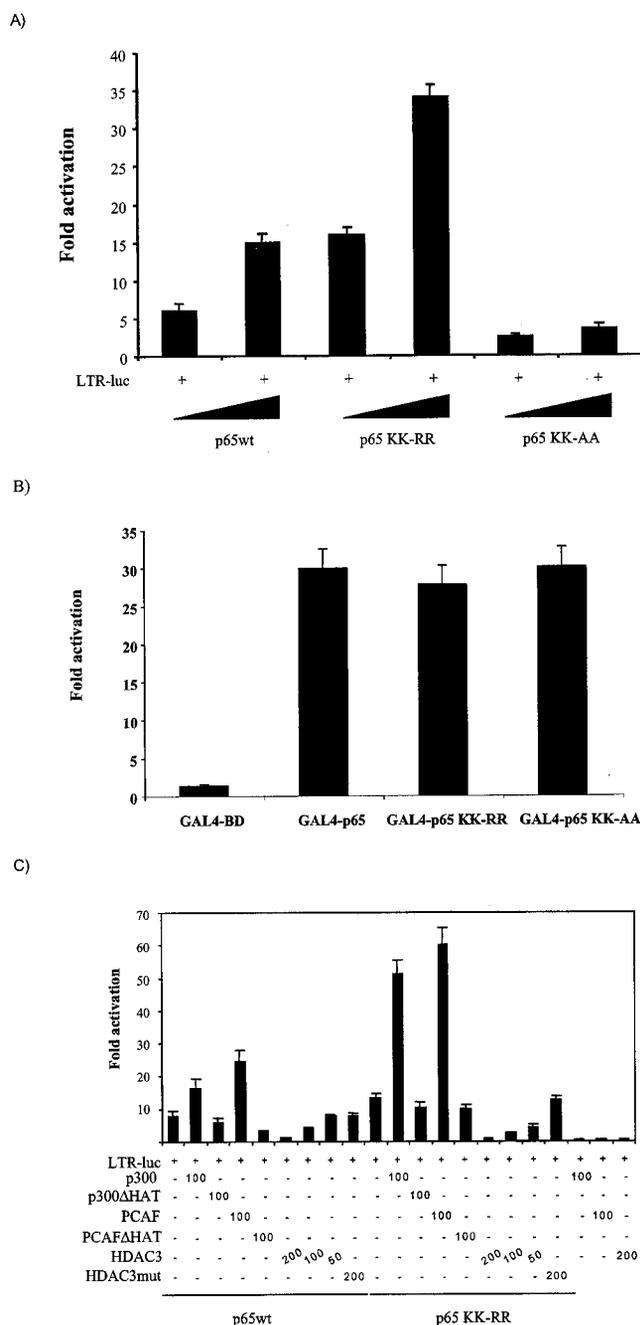
5D, panels 1 and 2). Induction of endogenous IκBα by p65 led to its export to the cytoplasm (data not shown). Cotransfection of p65 and IκBα led to the export of p65 to the cytoplasm in the

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 transactivation 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



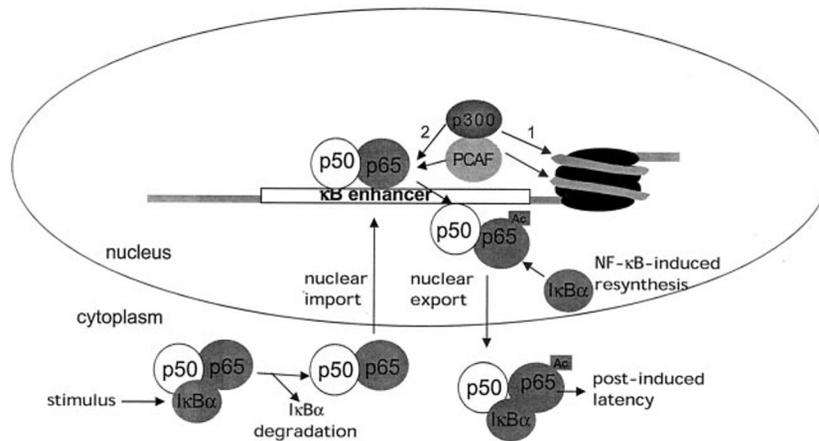


FIG. 8. Schematic model for the proposed role of p300- and PCAF-induced acetylation of p65 in regulating NF- κ B-mediated transcription. See text for details.

the KK-RR mutant (Fig. 7A) suggesting that acetylation of p65 negatively regulates its transcriptional activity and may contribute to the attenuation of p65 activity to restore the original latent state.

I κ B α -mediated removal of wild type and non-acetylated forms of p65 from the NF- κ B elements associated with the IL-8 promoter *in vivo* was assessed by ChIP analysis (32, 33). ChIP assay using anti-FLAG antibody was performed on cells transfected with FLAG-p65 or FLAG-p65KK-RR in the presence and absence of I κ B α . The IL-8 promoter intensities obtained from immunoprecipitates (Fig. 7B, IP, right panel) were first normalized to the starting chromatin extracts (Fig. 7B, input, left panel). The -fold enrichment was determined by dividing the normalized intensities of the transfected samples by that of the mock-transfected sample. ChIP analysis of cells transfected with FLAG-p65 (lane 13, upper panel) or FLAG-p65KK-RR (lane 16, upper panel) alone resulted in ~6- and 9-fold enrichment of sequences containing the IL-8 promoter, respectively. When I κ B α was cotransfected with wild type p65, 4- and 1.6-fold enrichment was observed, depending on the concentration of I κ B α transfected. However, 7.6- and 7-fold enrichment was observed when the same concentrations of I κ B α were cotransfected with p65KK-RR mutant. The expression levels of p65, p65KK-RR, and I κ B α are shown in Fig. 7C. Therefore, p65KK-RR is more resistant than wild type p65 to I κ B α -mediated removal from NF- κ B elements of the IL-8 promoter *in vivo*.

DISCUSSION

In this report, we examined the functions of p300 and PCAF coactivators in NF- κ B-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 κ B-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- κ B-mediated transcription. Following immune stimulation of cells, which results in degradation of I κ B α , NF- κ B is translocated to the nucleus via the newly exposed NLS in p65 and binds tightly to κ B-DNA elements. Recruitment of p300 and PCAF to the promoter region results in activation of NF- κ B-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 κ B-DNA. This facilitates removal of NF- κ B from enhancer elements by newly synthesized I κ B α whose expression is induced by NF- κ B, and

subsequent export of NF- κ B-I κ B α from the nucleus to the cytoplasm. The NF- κ B-I κ B α 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- κ B-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- κ B-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- κ B and I κ B α , 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 κ B DNA and thereby promotes I κ B α -mediated nuclear export. We were unable to demonstrate any significant effect of p65 acetylation on its interaction with I κ B α (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 κ B-DNA binding by acetylated and non-acetylated forms of p65 showed that acetylation of p65 lowered its affinity for κ B-DNA (Fig. 4), which consequently promotes its I κ B α -mediated export to the cytoplasm (Figs. 5, 7, and 8). Using a GST-I κ B α pull-down assay, Chen *et al.* showed that cotransfection of p300 and p65 reduces the ability of GST-I κ B α to interact with p65 and concluded that acetylation of p65 prevents its interaction with I κ B α . We directly analyzed binding of I κ B α to acetylated p65 by coimmunoprecipitation analysis using extracts from either [3 H]NaAc or [35 S]Met/Cys-labeled cells and found that most of the acetylated p65 in cells can be found in association with I κ B α . Indeed, given that acetylation of p65 occurs in the nucleus (Fig. 5), the finding that acetylated p65 accumulates in the cytoplasm in an I κ B α -dependent manner indicates that the interaction between p65 and I κ B α is not abolished, because studies using knock-out mice have shown that nuclear export of NF- κ B depends on its interaction with I κ B α . The discrepancy between the conclusions reached by the two studies may be due to differences in the experimental approaches used.

The importance of I κ B-mediated NF- κ B export from the nucleus and consequently the termination of NF- κ B-dependent transcription has been provided from studies using I κ B α knockout mice (36, 37). However, the mechanism by which I κ B α removes NF- κ B from DNA is unknown. The p50/p65 heterodimer has a particularly high affinity for κ B-DNA, between 10^{-10} and 10^{-13} (38, 39), whereas the affinity of I κ B α for the p50/p65 heterodimer is 10^{-9} (40). Thus, the NF- κ B/ κ B-DNA interaction would need to be destabilized so that I κ B can compete for the removal of NF- κ B from DNA. In agreement with this, Munshi *et al.* (41) found that I κ B α was not able to remove NF- κ B from DNA in the context of the INF- β enhanceosome. The crystal structure of the p50/p65 heterodimer bound to κ B-DNA revealed that, among p65 residues involved in DNA binding, lysines 122 and 123 are the only residues that contact the DNA in the minor groove (31). Our results show that acetylation of lysines 122 and 123 within p65 by p300 and PCAF reduces its affinity to κ B-DNA (Fig. 3). Thus, we propose a model in which acetylation of p65 plays a role in I κ B-mediated removal of NF- κ B from DNA and termination of NF- κ B-mediated transcription. In agreement with this model, the acetylated form of p65 accumulated in the cytoplasm in an I κ B α -dependent manner, and the p65KK-RR mutant was less sensitive to I κ B α -mediated termination of transcription and removal from κ B-DNA than wild type p65 (Fig. 7). Analogous to the function attributed to p65 acetylation in this report, acetylation of high mobility group I(Y) by CBP has been shown to destabilize its interaction with DNA, which disrupts the enhanceosome and turns off INF- β gene expression (41).

The activity of NF- κ B has been shown to be regulated by 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 HAT or HDAC activity (15). p65 binds to p300/CBP and PCAF (16–21). Protein kinase A phosphorylation of p65 enhances the p65/CBP interaction leading to increased NF- κ B-dependent gene expression (21). Furthermore, acetyltransferase activity of CBP/p300 and PCAF enhance NF- κ B transcriptional activity (19, 20). Here, we show that p300 and PCAF HAT activities potentiate p65 transcriptional activity, whereas their FAT activities are repressive. Factor acetylation of p65 negatively regulates its transcriptional activity by lowering binding to κ B-DNA and facilitating its removal and export by I κ B α . Additionally, HDAC3 was more repressive toward p65KK-RR than p65 wt, suggesting that factor deacetylase activity of HDAC3 is involved in activating p65 transcriptional activity, perhaps by enhancing p65 binding to κ B-DNA. Thus, p300 and PCAF acetyltransferase activities play a role in both transcriptional activation and post-induction turn-off of p65-mediated transcription. A similar functional duality has been previously demonstrated for high mobility group I(Y), which, together with other transcriptional activators, recruits HAT activity to the enhanceosome to stimulate transcription and serves itself as a target for acetylation leading to enhanceosome disassembly and turn-off of transcription (41).

The demonstration that p300 and PCAF are involved in both transcriptional activation and post-induction turn-off implies that their acetylation activity would need to be regulated in a temporal and substrate-dependent manner. What prevents p300 and PCAF from acetylating p65 immediately after their recruitment to the promoter? One possibility is that other substrates such as histones provide better targets for acetylation. In support of this, we have observed that acetylation of p65 by p300 and PCAF *in vitro* is ~100-fold less efficient than that of nucleosomes (data not shown). Alternatively, the substrate specificity of p300 and PCAF acetylation activity may be reg-

ulated by post-translational modifications. Therefore, recruitment of p300 and/or PCAF by κ B-DNA-bound p65 may lead first to chromatin derepression and transcriptional activation. Subsequently, p300 and PCAF may acetylate p65 to facilitate its removal from DNA by I κ B α and its export to the cytoplasm to terminate NF- κ B-mediated transcription.

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REFERENCES

- Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9**, 2723–2735
- Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16**, 225–260
- Chen, F. E., and Ghosh, G. (1999) *Oncogene* **18**, 6845–6852
- Pahl, H. L. (1999) *Oncogene* **18**, 6853–6866
- Karin, M. (1999) *Oncogene* **18**, 6867–6874
- Sachdev, S., Hoffmann, A., and Hannink, M. (1998) *Mol. Cell. Biol.* **18**, 2524–2534
- Tam, W. F., Wang, W., and Sen, R. (2001) *Mol. Cell. Biol.* **21**, 4837–4846
- Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R. T., Virelizier, J. L., and Dargemont, C. (1997) *J. Cell Sci.* **110**, 369–378
- Huang, T. T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1014–1019
- Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) *EMBO J.* **18**, 6682–6693
- Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999) *J. Biol. Chem.* **274**, 9108–9115
- Tam, W. F., Lee, L. H., Davis, L., and Sen, R. (2000) *Mol. Cell. Biol.* **20**, 2269–2284
- Le Bail, O., Schmidt-Ullrich, R., and Israel, A. (1993) *EMBO J.* **12**, 5043–5049
- Sun, S. C., Ganchi, P. A., Ballard, D. W., and Greene, W. C. (1993) *Science* **259**, 1912–1915
- Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) *Ann. Rev. Biochem.* **70**, 81–120
- Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2927–2932
- Hottiger, M. O., Felzien, L. K., and Nabel, G. J. (1998) *EMBO J.* **17**, 3124–3134
- Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275**, 523–527
- Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999) *Mol. Cell. Biol.* **19**, 6367–6378
- Vanden Berghe, W., De Bosscher, K., Boone, E., Plaisance, S., and Haegeman, G. (1999) *J. Biol. Chem.* **274**, 32091–32098
- Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1**, 661–671
- Chen, L., Fischle, W., Verdin, E., and Greene, W. C. (2001) *Science* **293**, 1653–1657
- Emiliani, S., Fischle, W., Van Lint, C., Al-Abed, Y., and Verdin, E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2795–2800
- Kiernan, R. E., Vanhulle, C., Schiltz, L., Adam, E., Xiao, H., Maudoux, F., Calomme, C., Burny, A., Nakatani, Y., Jeang, K. T., Benkirane, M., and Van Lint, C. (1999) *EMBO J.* **18**, 6106–6118
- Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11259–11263
- Benkirane, M., Chun, R. F., Xiao, H., Ogryzko, V. V., Howard, B. H., Nakatani, Y., and Jeang, K. T. (1998) *J. Biol. Chem.* **273**, 24898–24905
- Fajas, L., Paul, C., Vie, A., Estrach, S., Medema, R., Blanchard, J. M., Sardet, C., and Vignais, M. L. (2001) *Mol. Cell. Biol.* **21**, 2956–2966
- Nissen, R. M., and Yamamoto, K. R. (2000) *Genes Dev.* **14**, 2314–2329
- Furia, B., Deng, L., Wu, K., Baylor, S., Kehn, K., Li, H., Donnelly, R., Coleman, T., and Kashanchi, F. (2002) *J. Biol. Chem.* **277**, 4973–4980
- Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S., Jr. (2001) *Mol. Cell. Biol.* **21**, 7065–7077
- Chen, F. E., Huang, D. B., Chen, Y. Q., and Ghosh, G. (1998) *Nature* **391**, 410–413
- Braunstein, M., Sobel, R. E., Allis, C. D., Turner, B. M., and Broach, J. R. (1996) *Mol. Cell. Biol.* **16**, 4349–4356
- Orlando, V., Strutt, H., and Paro, R. (1997) *Methods* **11**, 205–214
- Bannister, A. J., Miska, E. A., Gorlich, D., and Kouzarides, T. (2000) *Curr. Biol.* **10**, 467–470
- Imhof, A., Yang, X. J., Ogryzko, V. V., Nakatani, Y., Wolffe, A. P., and Ge, H. (1997) *Curr. Biol.* **7**, 689–692
- Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995) *Nature* **376**, 167–170
- Klement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., Chen, C. H., Rosen, C. A., and Stewart, C. L. (1996) *Mol. Cell. Biol.* **16**, 2341–2349
- Thanos, D., and Maniatis, T. (1992) *Cell* **71**, 777–789
- Urban, M. B., and Baeuerle, P. A. (1990) *Genes Dev.* **4**, 1975–1984
- Phelps, C. B., Sengchanthalangsy, L. L., Huxford, T., and Ghosh, G. (2000) *J. Biol. Chem.* **275**, 29840–29846
- Munshi, N., Merika, M., Yie, J., Senger, K., Chen, G., and Thanos, D. (1998) *Mol. Cell* **2**, 457–467