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The Phosphorylation Status of Nuclear NF-κB Determines Its Association with CBP/p300 or HDAC-1

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Summary

Homodimers of the NF-κB p50 subunit are transcriptionally repressive in cells, whereas they can promote transcription in vitro, suggesting that their endogenous effects are mediated by association with other factors. We now demonstrate that transcriptionally inactive nuclear NF-κB in resting cells consists of homodimers of either p65 or p50 complexed with the histone deacetylase HDAC-1. Only the p50-HDAC-1 complexes bind to DNA and suppress NF-κB-dependent gene expression in unstimulated cells. Appropriate stimulation causes nuclear localization of NF-κB complexes containing phosphorylated p65 that associates with CBP and displaces the p50-HDAC-1 complexes. Our results demonstrate that phosphorylation of p65 determines whether it associates with either CBP or HDAC-1, ensuring that only p65 entering the nucleus from cytoplasmic NF-κB:IκB complexes can activate transcription.

Introduction

The transcription factor NF-κB, which is critical for inducible expression of many genes involved in immunity and inflammation (Ghosh et al., 1998), exists in the cytosol of resting cells bound to inhibitory IκB proteins. Stimulation with specific inducers, such as TNFα or LPS, activates an IκB kinase (IKK) complex that phosphorylates IκB, triggering its degradation by the proteasome (Karin and Ben-Neriah, 2000) and allowing free NF-κB to translocate to the nucleus and activate gene expression. We showed recently that cytoplasmic NF-κB:IκB complexes also contain the catalytic subunit of PKA (PKAc) that is maintained inactive (Zhong et al., 1997). However, IκB degradation activates this PKAc that phosphorylates the NF-κB p65 subunit at Ser 276 rendering NF-κB that enters the nucleus transcriptionally active through recruitment of CREB binding protein (CBP)/p300 (Zhong et al., 1998).

This critical role of histone acetylases in regulating the transcriptional activity of NF-κB has focused attention on acetylation in the overall context of NF-κB activation. The precise role of histone deacetylases (HDACs) in this process remains uncertain, though recent studies have shown that p65 interacts with distinct HDAC isoforms to negatively regulate gene expression (Ashburner et al., 2001; Chen et al., 2001; Ito et al., 2000; Lee et al., 2000). It is also not clear whether the interaction of NF-κB with acetylases or deacetylases is regulated, though recent evidence supports a role for phosphorylation in determining the transcriptional capacity of nuclear NF-κB. In addition to PKA, casein kinase II and IKK (Bird et al., 1997; Madrid et al., 2001; Wang et al., 2000) have been implicated in this process, although the relative importance of these has not been determined. Furthermore, deletion of GSK3β and TBK/T2K/NAK demonstrated that these kinases also affect NF-κB transcriptional activity (Bonnard et al., 2000; Hoeflich et al., 2000; Tojima et al., 2000), thereby explaining the embryonic lethality of these knockout strains that die from massive hepatocyte apoptosis similar to $p65^{-/-}$ or $IKK\beta^{-/-}$ mice (Beg et al., 1995; Li et al., 1999). More recently, deletion of NF-κB-inducing kinase (NIK) revealed that, although $NIK^{-/-}$ mice are viable, cells derived from them do not generate transcriptionally active NF-κB when stimulated with lymphotoxin β, while the response to all other NF-κB inducers is normal (Yin et al., 2001). The relationship of these separate pathways to PKA-mediated transcriptional activation remains to be elucidated; however, these studies provide the basis for systematic examination of the mechanisms through which distinct kinases influence the transcriptional activity of nuclear NF-κB.

The most abundant form of NF-κB in cells is a heterodimer of p50 and p65 that is retained in the cytosol by IκB (Ghosh et al., 1998); however, low levels of NF-κB, particularly p50 homodimers, can be detected in the nucleus of most unstimulated cells (Kang et al., 1992; Ten et al., 1992). Although p50 does not contain a transactivation domain and cannot recruit histone acetyltransferase (HAT) activity, it can interact with other nuclear proteins to promote gene transcription (Cha-Molstad et al., 2000; Fujita et al., 1993; Heissmeyer et al., 1999). For example, p50 interacts with Bcl-3, which recruits HATs to induce expression of κB-regulated genes (Dechend et al., 1999). However, the ability of p50 to decrease κB-dependant gene expression has also been demonstrated (Ledebur and Parks, 1995; Plaksin et al., 1993). Moreover, suppression of TNFα expression due to "endotoxin tolerance" is eliminated by p50 deficiency (Bohuslav et al., 1998), and a genetic polymorphism that prevents p50 homodimer binding to a κB site in the TNFα gene increases expression from that promoter (Udalova et al., 2000). Furthermore, a soluble factor released from macrophages induces p50 binding to the TNFα promoter, thereby decreasing TNFα expression (Baer et al., 1998).

The mechanism by which p50 homodimers inhibit gene expression is unknown, although it is unlikely that p50 subunits are intrinsically unable to drive transcription, as homodimers are active when tested in vitro (Fujita et al., 1992). It is believed that p50 homodimers compete for κB site binding with NF-κB complexes containing subunits such as p65 that have transactivation domains; however, this is unlikely to be their primary mechanism of repression, as their affinity for classical κB sites is lower than that of heterodimers such as p50:p65 (Kunsch et al., 1992; Phelps et al., 2000). One

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possible mechanism that has been demonstrated for other repressive DNA binding proteins (Glass and Rosenfeld, 2000) is that nuclear p50 may interact with separate inhibitory proteins that recruit corepressor complexes containing HDACs to gene promoters.

The studies we describe here demonstrate that nuclear NF-κB in unstimulated cells consists primarily of p50 homodimers associated with HDAC-1 that bind to DNA and repress NF-κB-dependent gene expression. Following stimulation, p50:p65 heterodimers containing phosphorylated p65 enter the nucleus and displace DNA-bound p50:HDAC-1, thereby providing a mechanistic explanation for repression by nuclear p50 homodimers in unstimulated cells and implicating acetylation and deacetylation as key regulatory processes for NF-κB-dependent transcription. Our results also demonstrate that phosphorylation of nuclear NF-κB determines whether it associates with CBP/p300 or HDAC-1, ensuring that only signal-induced NF-κB entering the nucleus can activate transcription.

Results

NF-κB-Dependent Transcription Requires Acetylases and Deacetylases

Our prior studies implicated CBP/p300 as a critical regulator of NF-κB activity and showed that recruitment of CBP was enhanced by phosphorylation of p65 at Ser 276 (Zhong et al., 1998). Consequently, to determine the contribution of the endogenous acetylase activity of CBP/p300 to NF-κB-dependent transcription, we used an inactive mutant (CBP-HAT$^-$) harboring the mutations L1690K and C1691L (Korzus et al., 1998). As reported previously (Zhong et al., 1998), cotransfection of CBP and PKAc significantly enhanced the transcriptional activity of wild-type p65 (Figure 1A), an effect that is not observed with the p65 S276A mutant (data not shown). Interestingly, this effect was attenuated when CBP-HAT$^-$ was used (Figure 1A), even though this mutant associates efficiently with phosphorylated p65 (data not shown). Previous workers suggested that CBP/p300-bound *p*300/*C*BP *a*ssociated *f*actor (P/CAF) regulates NF-κB transcriptional activity (Sheppard et al., 1999); however, we found no significant stimulation of p65-mediated transcription by P/CAF (instead it may even exert a slight inhibition), either alone or in the presence of CBP (Figure 1A).

This apparent role for acetylation in p65-dependent transcription led us to speculate that deacetylation might also modulate NF-κB activity. We therefore began our analysis by studying HDAC-1, one of the most abundant mammalian histone deacetylase isoforms (Taunton et al., 1996). To test whether HDAC-1 affects NF-κB-dependent transcription, we transfected Jurkat cells with HDAC-1 together with p65 and found that full-length HDAC-1 dose-dependently inhibited NF-κB (Figure 1B). In contrast, a version lacking deacetylase activity (residues 193–450) had no effect, whereas an NH_2-terminal fragment (residues 1–192) including the deacetylase domain inhibited NF-κB-dependent transcription (Figure 1B). This demonstrates that increasing the concentration of histone deacetylase in cells inhibits p65-dependent transcription. To test the effect of HDAC-1 on transcription mediated by endogenous NF-κB, we transfected Jurkat cells prior to stimulation with PMA/PHA, and similar to the results above (Figure 1B), full-length HDAC-1 and HDAC-1 (1–192) strongly inhibited NF-κB activity (Figure 1C).

To exclude the possibility that the results of Figures 1A–1C were due to effects of overexpressing CBP, HDAC-1, or PKAc on the levels of p65 expression, we immunobloted transfected cell extracts using antibodies against the HA epitope on the transfected p65. As shown in Figure 1D, neither CBP, HDAC-1, nor PKAc affected p65 levels in transfected cells.

Nuclear NF-κB in Unstimulated Cells Is Associated with HDAC-1

We next determined whether interactions occurred between endogenous HDAC-1 and NF-κB proteins. The majority of NF-κB in unstimulated cells is localized to the cytoplasm, but low levels of p50 and even lower levels of p65 can be detected in the nucleus (Figure 2A). Since HDAC-1 is exclusively nuclear (Taunton et al., 1996), we surmised that if NF-κB proteins associated with HDAC-1 in unstimulated cells, these most likely form transcriptionally inactive nuclear complexes. We therefore immunoprecipitated HDAC-1 from nuclear extracts of resting cells and found that it did indeed associate with both p50 and p65 (Figure 2B, left panel) via noncovalent interactions disrupted by boiling the extracts. Reciprocal experiments using immunoprecipitating antibodies against p50 and p65 also demonstrated the association of HDAC-1 with both proteins (Figure 2B, right panel).

To verify these interactions using overexpressed proteins, we transiently transfected HEK293 cells with p50 or p65 together with HDAC-1 and immunoprecipitated the proteins from lysates. Following immunoblotting, we detected efficient interactions between HDAC-1 and p50 or p65 in these transfected cells (Figure 2C). We next asked whether NF-κB proteins interact directly with HDAC-1 or whether this requires adaptor molecules such as Sin3, which is known to associate with HDAC-1 (Laherty et al., 1997; Nagy et al., 1997). To our surprise, after probing the immunoblots from Figure 2C with antibodies against Sin3, we found that while the HDAC-1 immunoprecipitates contained low amounts, neither p50 nor p65 immunoprecipitates contained detectable levels of Sin3 (data not shown). We therefore incubated GST fusion proteins of p50 and p65 with in vitro translated HDAC-1 and found that both GST-p50 and the GST-p65 Rel domain (amino acids 1–313) interacted with HDAC-1 in vitro (Figure 2D, lanes 1 and 2). Interestingly, GST-p65 (1–313) phosphorylation by PKA significantly lowered the efficiency of interaction with HDAC-1 (Figure 2D, lane 5 versus 2), and consistent with this, a p65 mutant that cannot be phosphorylated by PKA (p65^{mt} [1–313]) remained associated with HDAC-1 even after incubation with PKA (Figure 2D, compare lanes 7 and 6). We also performed the reciprocal experiment and found that GST-HDAC-1 efficiently precipitated p50 and p65 (Figure 2D, lanes 8 and 10). Taken together, these findings strongly suggest that, unlike other transcription

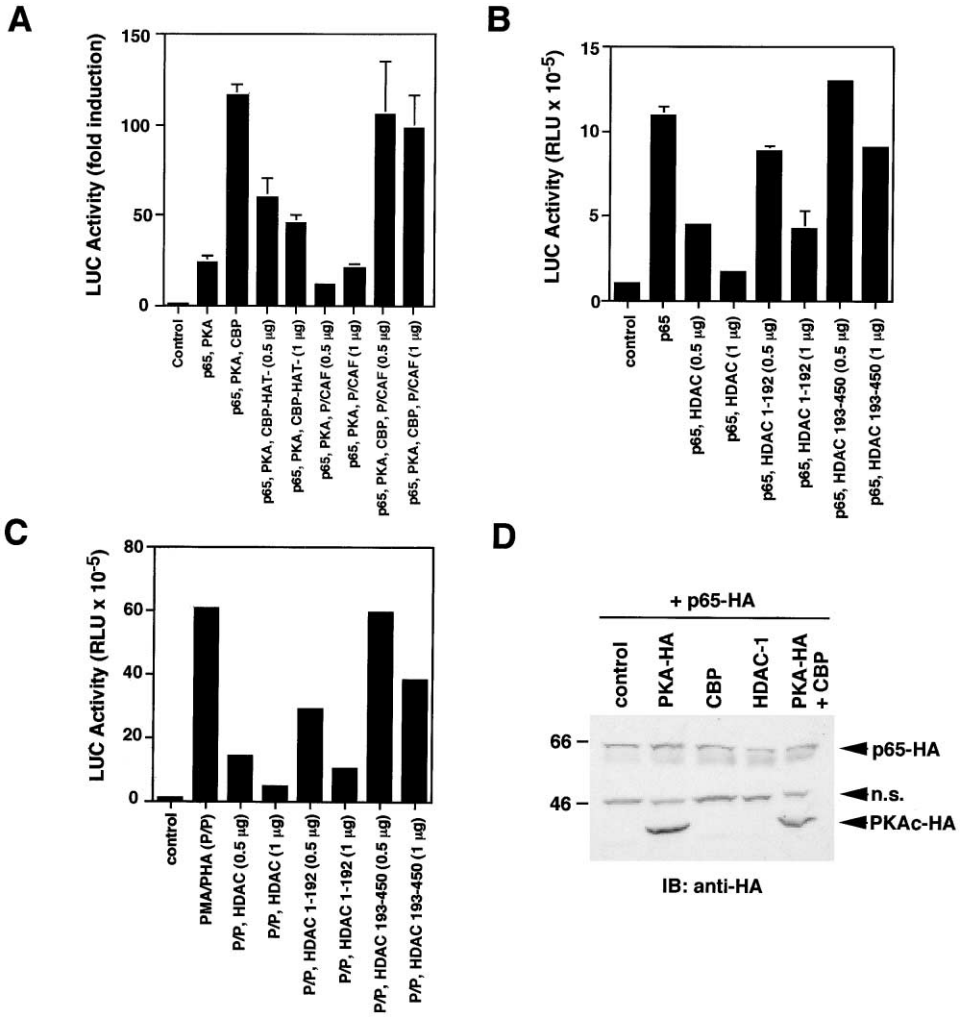

Figure 1. Acetylation and Deacetylation Regulates NF-κB Transcriptional Activity

(A) Jurkat cells were transfected with the κB-dependent reporter construct pBIIx-luc and the indicated plasmids. Cells were harvested 24 hr later and assayed for luciferase activity that is reported as fold induction compared with pBIIx-luc alone (control).

(B) Jurkat cells were transfected with pBIIx-luc and either p65 alone or together with wild-type or truncated HDAC-1 (1–192 or 193–450). Luciferase assays were performed 24 hr later.

(C) Jurkat cells were transfected for 24 hr with pBIIx-luc and the HDAC-1 fragments shown and then stimulated for 4 hr with PMA/PHA (P/P) prior to luciferase assay.

(D) Cells were transfected with the plasmids shown (top), then lysates were immunoblotted (IB) with anti-HA. p65-HA, PKAc-HA, and a nonspecific band (n.s.) are indicated.

factors that associate with deacetylase complexes via adaptor proteins, NF-κB interacts directly with HDAC-1.

Nuclear p50-HDAC-1 Complexes in Unstimulated Cells Bind to DNA

The interaction between nuclear NF-κB subunits and HDAC-1 in unstimulated cells (Figure 2B) led us to question whether such complexes could bind to DNA. We therefore performed electrophoretic mobility shift assays (EMSA) with a specific κB probe and consistently observed a DNA-protein complex (complex C2, Figure 3A) in nuclear extracts from unstimulated cells. Stimulation with PMA/PHA led to the appearance of a slower migrating complex (complex C1) along with somewhat enhanced levels of the faster migrating C2 complex (Figure 3A). The C2 complex is specific for the κB site since an unlabeled oligonucleotide with the wild-type κB sequence, but not a mutated oligonucleotide harboring two G to A substitutions, effectively competed for binding (Figure 3B, lanes 2–4 versus lanes 5–7).

The EMSA in Figure 3A was performed under standard conditions using excess κB probe, although it is unlikely that the concentration of target κB sites in cells is in vast excess. We therefore repeated the experiment using limiting amounts of probe, reasoning that under these conditions, the complex with the greater affinity would bind preferentially. This was particularly relevant since it has been demonstrated that p50 homodimers bind to canonical κB sites, such as the Igκ enhancer sequence used as our probe, with lower affinity compared with

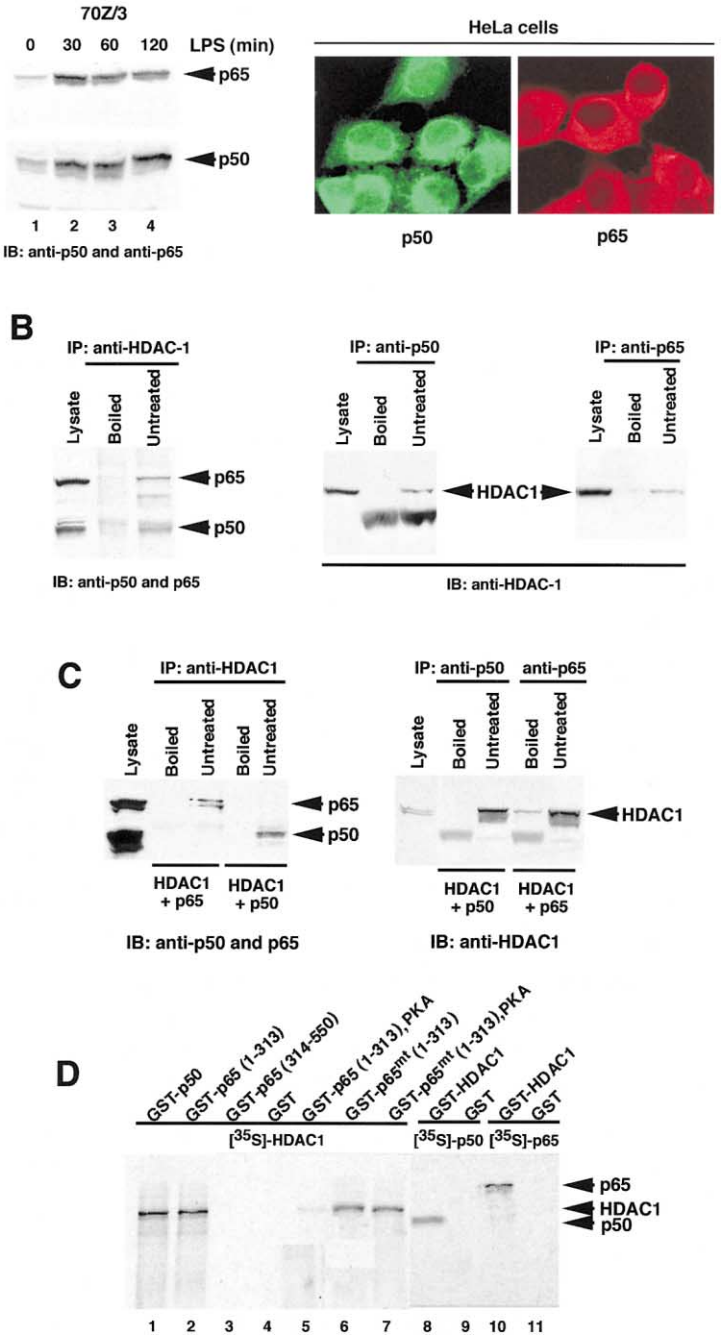

Figure 2. HDAC-1 Associates with Nuclear p65 and p50 In Vivo

(A) Nuclear extracts from resting or LPS-stimulated (10 μg/ml) 70Z/3 cells were immunoblotted (IB) using anti-p65 and anti-p50 (left). Immunofluorescence in HeLa cells was detected using the indicated antibodies localized with FITC (p50) or Texas Red (p65) (right). Samples with the secondary antibody alone showed no nuclear staining (data not shown).

(B) Nuclear extracts from resting 70Z/3 cells were immunoprecipitated (IP) using the antibodies shown (top). Identical samples were boiled with SDS and neutralized with Triton X-100 before adding antibodies (Boiled). Immunoprecipitates and lysates from transfected cells were immunoblotted (IB) with the antibodies shown (bottom).

(C) HEK293 cells were transfected as indicated below each panel and immunoprecipitations (IP) were performed using the antibodies shown (top). Lysates and boiled controls precipitated with the same antibodies were run in parallel, and immunoblots were probed as shown (IB; bottom).

(D) In vitro-translated [^{35}S]HDAC-1, p50, and p65 were mixed with the GST proteins shown. Two samples (lanes 5 and 7) were incubated with PKA before mixing with HDAC-1. After incubation (22°C; 15 min), proteins were precipitated using glutathione-agarose, electrophoresed, and then visualized by fluorography.

heterodimeric complexes such as p50:p65 (Kunsch et al., 1992; Phelps et al., 2000). As shown in Figure 3C, under limiting probe conditions, the C2 complex is abolished, and the heterodimer C1 complexes are the preferred κB site binders in stimulated cells. This therefore demonstrates that signal-induced heterodimeric complexes entering from the cytoplasm likely displace the C2 complexes in resting cells.

To characterize the components of the C2 complex from unstimulated cells, we performed supershift analysis, and as shown in Figure 3D, the C2 complex was almost completely abolished by antibodies against p50 but not p65 (Figure 3D, lane 4 versus lane 6). More interestingly, antibodies against HDAC-1 (lane 2) also blocked this complex, suggesting that it consists of both p50 and HDAC-1. Despite multiple attempts, we never observed a supershifted complex using anti-HDAC-1, raising the possibility that inhibition of the C2 complex was due to nonspecific interference from the antibody we used. We therefore tested two additional HDAC antibodies, and both of these also inhibited DNA binding of the C2 complex without generating a supershifted complex (Figure 3D, lanes 9 and 10). We also performed assays using a peptide that blocked binding of the Santa Cruz antibody and verified the specificity for HDAC-1 (data not shown). No such blocking peptide is available

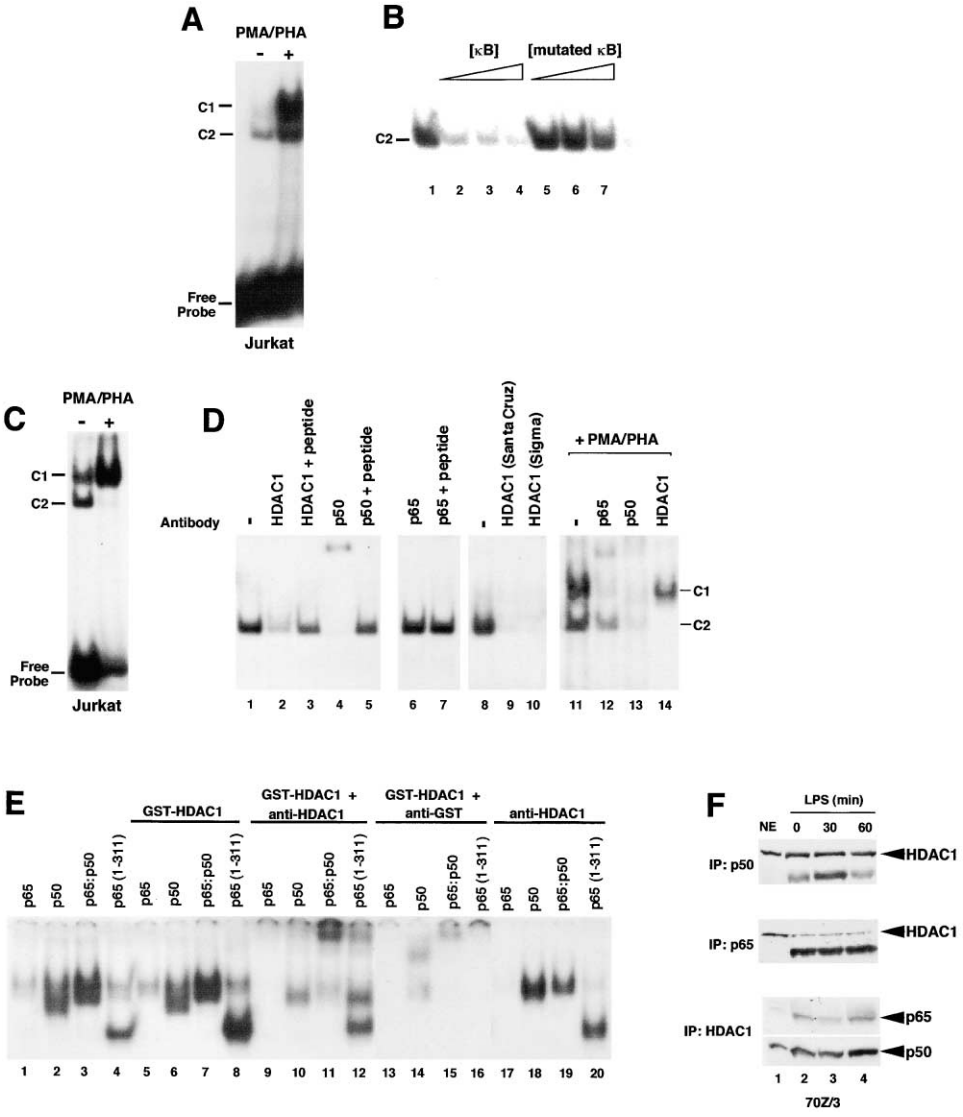

Figure 3. HDAC-1 Forms Complexes with p50 Homodimers in the Nuclei of Resting Cells

(A) Nuclear extracts from untreated or PMA/PHA-treated Jurkat cells were analyzed by EMSA.

(B) Nuclear extracts from untreated Jurkat cells were used for EMSA. Reactions contained labeled κB probe alone (lane 1) or together with 25, 50, or 100 ng (lanes 2, 3, and 4, respectively) of unlabeled or mutated κB probe (lanes 5, 6, and 7).

(C) The samples from the experiment in (A) were reanalyzed by EMSA using 1/10 of the amount of κB probe.

(D) Supershift analysis of κB-DNA binding complexes in resting cells (lanes 1–10) was performed using anti-HDAC-1, anti-p65, or anti-p50 as indicated (top). The blocking peptides were used to establish specificity of the antibodies (lanes 3, 5, and 7). In lanes 11–14, nuclear extracts were made from PMA/PHA-treated cells (30 min). The positions of the C1 and C2 complexes are shown.

(E) Homodimeric p50 or p65, p50:p65 heterodimers, and p65 (1–311) were made by in vitro translation and used for EMSA (lanes 1–4). In lanes 5–16, samples were preincubated with GST-HDAC-1 (30 min; 4°C), and supershift analysis was performed using anti-HDAC-1 (lanes 9–12 and 17–20) or anti-GST (lanes 13–16).

(F) Proteins in nuclear extracts from resting or LPS-treated 70Z/3 cells were immunoprecipitated (lanes 2–4) using the antibodies indicated (left; IP). A sample of untreated extract (NE; lane 1) was run with the precipitates, and resulting immunoblots were probed using the antibodies shown (right).

for the Sigma antibody, but in light of our findings using these multiple reagents, we are confident that its effects are directly attributable to HDAC-1 binding. As a control, we tested the anti-HDAC-1 antibodies on the p50:p65 heterodimer from PMA/PHA-stimulated cells, and while anti-p50 and anti-p65 antibodies inhibited the C1 complex (p50:p65), anti-HDAC-1 had no effect (lanes 11–14). In contrast, both anti-HDAC-1 and anti-p50 inhibited the C2 complex (p50:p50), whereas anti-p65 had little effect. Taken together, these data demonstrate that in unstimulated nuclear extracts, the C2 complex contains p50 homodimers associated with HDAC-1.

We next examined the interaction of HDAC-1 with NF-κB using recombinant proteins in EMSAs. As reported previously (Zhong et al., 1998), p65 bound very poorly to κB sites whereas p50 homodimers, p50:p65 hetero-

dimers, and the NH_2-terminal DNA binding fragment of p65 (residues 1–311) bound efficiently (Figure 3E, lanes 1–4). Addition of recombinant GST-HDAC-1 prior to DNA binding did not significantly alter the migration of the p50 and p50:p65 DNA-protein complexes (Figure 3E, lanes 6 and 7 versus lanes 2 and 3), and similar to the results with the endogenous C2 complex (Figure 3D), the anti-HDAC-1 antibody blocked DNA binding of the p50:GST-HDAC-1 complex without generating a visible supershifted complex (Figure 3E, lane 10). However, the p50:p65:GST-HDAC-1 complex (Figure 3E, lane 11) and the p65 (1–311) homodimer:GST-HDAC-1 complex (Figure 3E, lane 12) did generate weakly supershifted complexes with anti-HDAC-1. To further establish the presence of GST-HDAC-1 in the complexes in lanes 6–8, we used anti-GST (Figure 3E, lanes 13–16) and, similarly to anti-HDAC-1, this abolished DNA binding of the complexes without yielding strong supershifted bands. When tested alone, neither anti-HDAC-1 (Figure 3E, lanes 17–20) nor anti-GST (data not shown) affected the DNA-protein complexes formed in the absence of GST-HDAC-1.

The experiments in Figure 3 suggest that p50-HDAC-1 but not p65-HDAC-1 complexes bind to DNA in unstimulated nuclear extracts. One reason why p65-HDAC-1 complexes may not associate with DNA is that full-length, unphosphorylated p65 binds poorly to DNA (Zhong et al., 1998), and as nuclear p65 in resting cells is unlikely to be phosphorylated, this represents an additional regulatory function for phosphorylation of p65. Thus, in addition to promoting CBP recruitment, phosphorylation may also regulate DNA binding of nuclear p65, ensuring that p65 homodimers in resting cells do not promote unwanted transcription. Our finding that p50 homodimers are displaced by heterodimer complexes entering the nucleus after stimulation (Figure 3C) led us to surmise that nuclear p50- and p65-HDAC-1 complexes exist as separate signal-independent pools. To test this, we treated 70Z/3 cells with LPS and found that the p50- and p65-HDAC-1 complexes are maintained after stimulation (Figure 3F). We also probed identical cytoplasmic extracts using anti-IκBα and observed complete degradation after 60 min with LPS (data not shown), ruling out the possibility that only some of the cells respond to LPS and that the immunoprecipitated complexes (Figure 3F) are from unstimulated cells. This result is therefore consistent with our model in which nuclear NF-κB-HDAC-1 complexes are nonregulated pools that remain unaltered even after p50-HDAC-1 is displaced from DNA by activated p50:p65 heterodimers.

DNA-Bound p50-HDAC-1 Suppresses NF-κB-Dependent Gene Expression

Our findings suggesting that p50-HDAC-1 complexes bind DNA in unstimulated cells may explain why baculovirus-produced p50 homodimers that do not activate transcription in cells are transcriptionally active in vitro (Fujita et al., 1992; Schmitz and Baeuerle, 1991). If such inhibition in cells occurred via association of HDAC-1 with p50, we reasoned that inhibition of HDAC-1 activity in unstimulated cells would activate DNA-bound p50, causing κB-dependent transcription. To test this, we used the specific HDAC inhibitor trichostatin A (TSA; Hassig et al., 1997) and found that it reverses inhibition of κB-dependent transcription by transfected HDAC-1 (Figure 4A). To determine whether DNA-bound p50 homodimers were repressive due to associated HDAC-1, we generated stable cell lines that express pBIIx-luc, and as a control, we made lines with pFluc that lacks the two κB sites (Kopp et al., 1999). After treatment of both cell lines with TSA (20 nM), only the pBIIx-luc reporter was time-dependently activated (Figure 4B), strongly suggesting that nuclear NF-κB in resting cells binds to κB sites but does not activate transcription due to association with HDAC-1. It is possible that the slow kinetics of TSA-activated transcription compared with inducers such as TNFα or IL-1 may be due to a lack of nuclear p50 phosphorylation, as we have demonstrated that CBP/p300 recruitment to p65 requires phosphorylation. Since nuclear p50 in TSA-treated cells is not associated with p65 and is presumably unphosphorylated, it is unlikely to exhibit the strong transcriptional activity obtained by association of factors such as CBP/p300.

We next examined the effects of TSA on endogenous κB-dependent gene expression, having speculated that transcriptional activation of DNA-bound p50 by HDAC inhibition would be unlikely to upregulate all κB-dependent genes. It is known that very few genes are completely dependent on a single transcription factor and that binding of multiple factors is required for robust activation of most natural promoters. Furthermore, it is unlikely that p50 homodimers recruit CBP/p300, which is necessary for the full transcriptional activity of NF-κB (see above). Despite these potential drawbacks, we treated HEK293 cells with TSA and analyzed expression of selected κB-dependent (IL-8 and TNFβ) and -independent (TGFβ, L32, and GAPDH) genes by RNase protection assay (RPA). Interestingly, only IL-8 and TNFβ were strongly induced by TSA (Figure 4C), although we observed slightly increased expression of various TGFβ isoforms (right panel), suggesting that these are controlled by constitutive HDAC activity recruited by transcription factors other than NF-κB.

Induction of IL-8 and TNFβ by TSA is consistent with our hypothesis that transcriptionally inactive p50-HDAC-1 binds to κB-dependent promoters in unstimulated cells; however, to unequivocally demonstrate the involvement of p50, we examined gene expression in cells from $p50^{-/-}$ mice. We first isolated peripheral blood lymphocytes (PBLs) from wild-type mice, treated them with PMA/ionomycin (P/I), TNFα, or LPS, and then examined expression of two κB-dependent (IL-6 and iNOS) and -independent (TGFβ and HPRT) genes by RT-PCR. As expected, IL-6 and iNOS were induced by all stimuli (Figure 4D, left panel). In contrast, LPS-induced expression of both was severely impaired in $p50^{-/-}$ cells while TGFβ and HPRT expression was unaffected (data not shown). We next treated PBLs with TSA and observed significantly increased expression of IL-6 and iNOS but not TGFβ or HPRT in wild-type cells (Figure 4D, right panel). Remarkably, TSA-induced IL-6 and iNOS expression was completely absent in $p50^{-/-}$ cells, clearly demonstrating that p50-HDAC-1 suppresses expression of certain genes in unstimulated cells. Interestingly, basal expression of iNOS was elevated in $p50^{-/-}$ cells, again consistent with a repressive role for p50.

Consistent with a model of nuclear p50-HDAC-1 com-

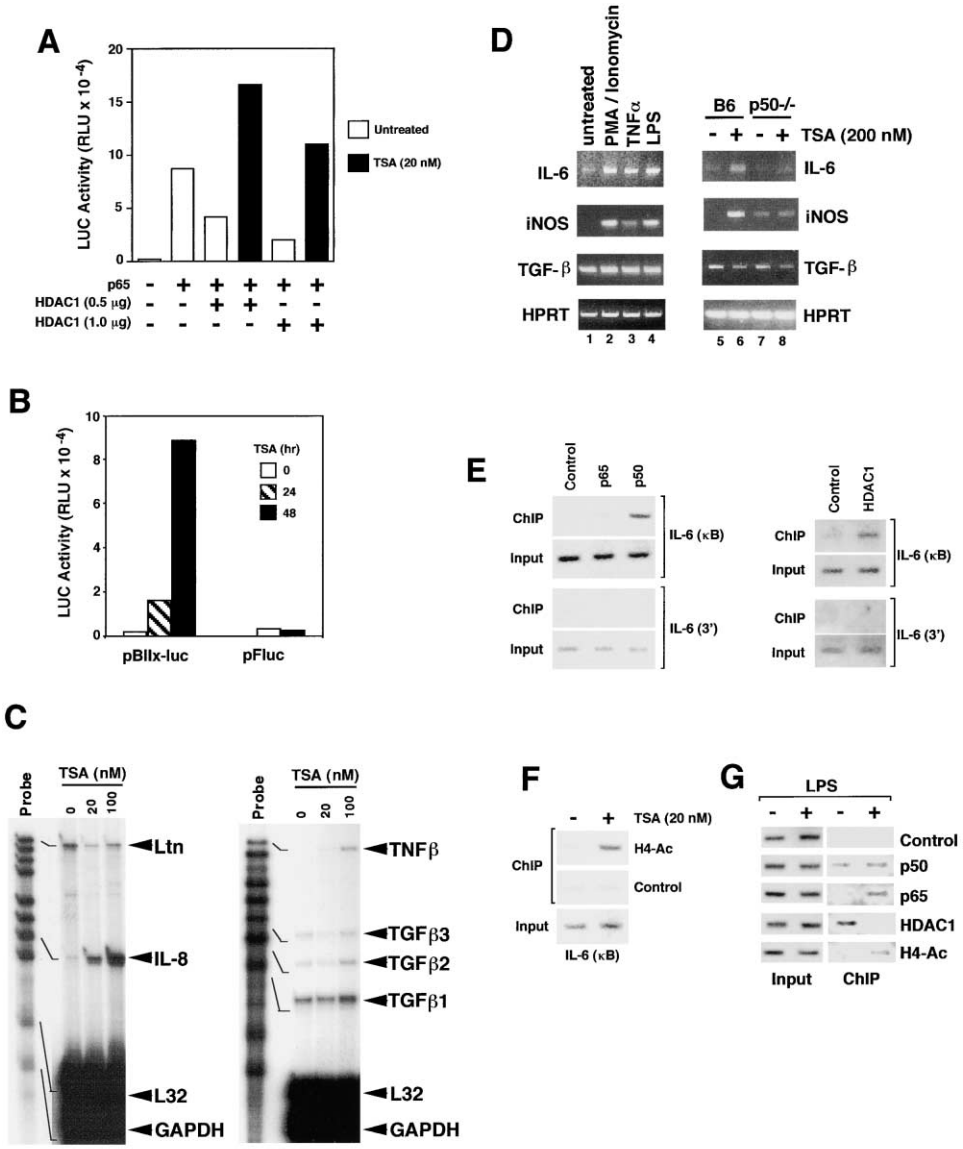

Figure 4. Inhibition of Histone Deacetylase Activity Induces NF-κB-Dependent Gene Expression in the Absence of Stimulation

(A) HEK293 cells were transfected (24 hr) with pBIIx-luc and p65 alone or together with HDAC-1 (0.5 or 1 μg) and then incubated (24 hr) in the absence (open bar) or presence (closed bar) of TSA (20 nM) before luciferase activity in extracts was measured.

(B) HEK293 stable cell lines harboring either pBIIx-luc or pFluc were treated with TSA (20 nM) for the times shown, and then extracts were assayed for luciferase activity.

(C) Total RNA was isolated from HEK293 cells treated (24 hr) with increasing doses of TSA, and then transcription was analyzed by RPA using human cytokine template sets 3 (left) and 5 (right) from Pharmingen.

(D) Thymocytes and splenocytes from wild-type B6 mice were treated with the stimuli indicated and then analyzed by RT-PCR using primers specific for the cytokine genes shown (left). HPRT levels were measured to ensure equal RNA loading. Thymocytes from wild-type B6 or $p50^{-/-}$ mice were treated with 200 nM TSA (12 hr), and then RNA was isolated and analyzed by RT-PCR (right).

(E) Chromatin from resting HEK293 cells was used for ChIP with protein G Sepharose alone (control), anti-p65, anti-p50, or anti-HDAC-1 as shown (top). Precipitated DNA was analyzed by PCR using primers specific for the IL-6 κB site or a 3′ region of the gene. PCR was also performed using 1% of input chromatin to ensure equal loading.

(F) Chromatin from resting or TSA-treated (20 nM; 24 hr) HEK293 cells was precipitated using either anti-acetylated H4 (H4-Ac) or protein G Sepharose alone (control). IL-6 promoter DNA was amplified by PCR, and PCR was also performed using the input (1%; bottom) to ensure that equal amounts were used for each sample.

(G) Chromatin from untreated or LPS-treated (10 μg/ml; 3 hr) RAW 264.7 cells was used for ChIP with protein G Sepharose (control) or the antibodies shown (right). Precipitated IL-6 κB site DNA was assayed by PCR (ChIP), and 1% of the chromatin was assayed to verify equal loading (Input).

plexes binding to κB sites in gene promoters (Figures 4C and 4D), previous workers demonstrated that p50 but not p65 associated with the IFN-β promoter in resting cells (Parekh and Maniatis, 1999; Senger et al., 2000). Therefore, to prove that p50 and HDAC-1 were associated with a TSA-regulated κB-dependent gene, we used chromatin immunoprecipitation analysis (ChIP) to investigate the proteins bound to the IL-6 promoter. In resting cells, p50 but not p65 was readily detected bound to the promoter (Figure 4E, left panel), whereas neither protein bound to DNA from a region within the 3′ end of the IL-6 gene, demonstrating specificity of p50 for the κB site. Consistent with our hypothesis, we also found that only the IL-6 κB site DNA coprecipitated with HDAC-1 (Figure 4E, right panel). We next determined whether TSA affected acetylation of IL-6 promoter-associated histones, as this correlates with increased transcription of many genes (Struhl, 1998), and acetylated histone H4 (H4-Ac) has been shown to associate with the IL-6 promoter following transcriptional activation (Saccani et al., 2001). As shown in Figure 4F, H4-Ac was not associated with the IL-6 gene in resting cells, whereas TSA treatment induced acetylation of IL-6-associated H4.

Our ChIP analyses clearly demonstrate that endogenous p50-HDAC-1 associates with the IL-6 promoter and that TSA treatment causes acetylation of associated H4. We therefore sought to determine the effects of a known NF-κB activator on the association of p50, p65, HDAC-1, and H4-Ac with the IL-6 κB site. Previous workers demonstrated that H4 associated with the IL-6 gene becomes acetylated with slow kinetics (Saccani et al., 2001), so we performed our analysis using chromatin from RAW264.7 cells treated with LPS for 3 hr. As shown in Figure 4G, the levels of IL-6 gene-associated p50 were unchanged in response to LPS, whereas those of p65 and H4-Ac were increased and the amount of associated HDAC-1 was decreased.

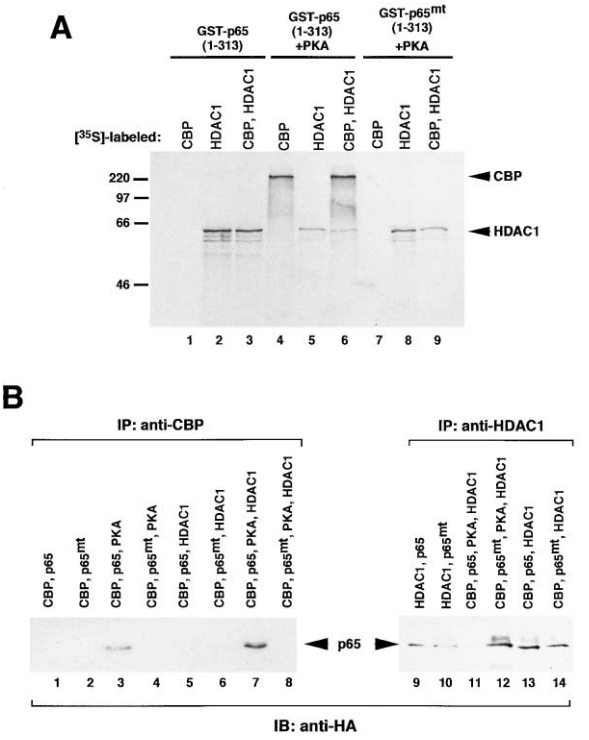

Figure 5. Phosphorylation of p65 by PKA Determines Whether It Binds HDAC-1 or CBP

(A) In vitro-translated and [35S]HDAC-1 or CBP were mixed with wild-type GST-p65 (1–313) or a mutant harboring the PKA phosphorylation site mutation S276A (p65mt). GST proteins were either unphosphorylated or phosphorylated in vitro by preincubation with PKA. Complexes were precipitated using glutathione-agarose, electrophoresed, and then analyzed by fluorography.

(B) COS cells were transfected with HA-tagged p65 or p65mt together with the combinations of CBP, HDAC-1, and PKA indicated (top). After 24 hr, immunoprecipitations (IP) were performed using anti-CBP (left) or anti-HDAC-1 (right), and then p65 was detected by immunoblotting with anti-HA.

Phosphorylation of p65 Determines Whether It Associates with CBP/p300 or HDAC-1

Signal-induced p65 phosphorylation by PKA regulates NF-κB transcriptional activity by recruiting CBP/p300 (Zhong et al., 1998); however, in resting cells both CBP/p300 and HDAC-1 are exclusively nuclear (Taunton et al., 1996). Consequently, only activated NF-κB that enters the nucleus and whose p65 subunit is phosphorylated can associate with CBP/p300. We have now demonstrated that in resting cells, the low level of most likely unphosphorylated constitutively nuclear p65 associates with HDAC-1, suggesting that phosphorylation determines whether nuclear p65 associates with HDAC-1 or CBP/p300. To demonstrate a direct role for phosphorylation in regulating this, we tested the ability of CBP and HDAC-1 to associate with GST-p65 (1–313) in vitro. As shown in Figure 5A, unphosphorylated GST-p65 (1–313) interacted efficiently with HDAC-1 but not CBP (Figure 5A, lanes 1 and 2), and when both CBP and HDAC-1 were mixed in equal amounts before adding GST-p65 (1–313), only HDAC-1 associated with p65 (Figure 5A, lane 3). To test the effect of p65 phosphorylation, the GST-p65 protein was incubated with PKA prior to use, and under these conditions, phosphorylated p65 associated strongly with CBP and poorly with HDAC-1 (Figure 5A, lanes 4 and 5 versus lanes 1 and 2). More remarkably, when phosphorylated GST-p65 was incubated with a mixture of HDAC-1 and CBP, it associated predominantly with CBP (Figure 5A, lane 6 versus 3). As expected, a mutant containing a S276A substitution (p65mt) associated mainly with HDAC-1 (not shown), and its preference for HDAC-1 was unaltered by PKA (Figure 5A, lanes 7–9).

To determine whether this effect of PKA-mediated p65 phosphorylation could be observed in cells, we made extracts from COS cells transfected with CBP, HDAC-1, PKA, and either wild-type or S276A (p65mt) versions of HA-tagged p65 (Figure 5B). Following immunoprecipitation using anti-CBP or anti-HDAC-1, we immunoblotted using anti-HA to detect p65. Stable association between wild-type p65 and CBP was only detected when PKA was cotransfected, whereas HDAC-1 associated with both wild-type and mutant p65 (Figure 5B, lanes 9 and 10). Most dramatically, in the presence of PKA, CBP, and HDAC-1, wild-type p65 associated with CBP but not HDAC-1 (Figure 5B, compare lanes 7 and 11), whereas p65mt only associated with HDAC-1 (Figure 5B,

compare lanes 8 and 12). These results clearly demonstrate a critical role for p65 phosphorylation in determining whether nuclear NF-κB associates with HDAC-1 or CBP/p300.

Discussion

We previously demonstrated that PKA-dependent p65 phosphorylation regulates the transcriptional activity of NF-κB (Zhong et al., 1997, 1998), suggesting that this is as important as IκB degradation for NF-κB-dependent gene expression. Other groups have also suggested that phosphorylation by casein kinase II and IKKβ (Bird et al., 1997; Madrid et al., 2001; Wang et al., 2000) plays a role, and clearly, further work will determine the relative contributions of each of these mechanisms. More surprisingly, characterization of embryos lacking GSK3β or TBK/T2K/NAK (Bonnard et al., 2000; Hoeflich et al., 2000; Tojima et al., 2000) demonstrated death from hepatocyte apoptosis similarly to $p65^{-/-}$ animals (Beg et al., 1995). Interestingly, κB-dependent transcription was reduced in cells lacking these kinases while nuclear translocation and DNA binding of NF-κB was normal. Although the mechanism underlying these effects is unknown, these findings support the hypothesis that phosphorylation of specific subunits regulates the transcriptional activity and biological function of NF-κB.

Why is an additional layer of regulation through posttranslational modification of p65 necessary? It was assumed that cytosolic partitioning of NF-κB:IκB complexes ensured that NF-κB is transcriptionally active only after entering the nucleus; however, recent findings show that NF-κB localization is dynamic, with the default location being the nucleus, and that a nuclear export sequence in IκBα promotes predominantly cytoplasmic localization (Huang et al., 2000). Therefore, a steady state of low levels of NF-κB is continually present in the nucleus, necessitating a mechanism to ensure that it cannot activate transcription. Signal-induced cytosolic p65 phosphorylation provides an elegant solution to this problem, as it ensures that only NF-κB activated by IκB degradation can activate transcription, thereby maintaining NF-κB as an inducible transcription factor.

Our results lead us to conclude that nuclear p65 homodimers are transcriptionally inactive, as unphosphorylated p65 does not bind DNA. Consequently, we do not believe that p65-HDAC-1 plays a direct role in suppressing basal NF-κB activity. Recent reports indicate that p65-associated HDAC regulates induced NF-κB activity but fail to define a mechanism by which p65 switches between association with CBP/p300 and HDAC. Furthermore, the precise complement of p65-interacting HDAC isoforms remains unclear, although we have rigorously demonstrated association with HDAC-1 via immunoprecipitation of endogenous and overexpressed proteins, a finding supported by others (Lee et al., 2000; Ashburner et al., 2001). However, separate reports of HDAC-3 (Chen et al., 2001) and HDAC-2 (Ito et al., 2000) interacting with p65 demonstrate its capacity to bind directly or indirectly to distinct HDACs. It is important to note that in our model, nuclear p65-HDAC-1 complexes in unstimulated cells are bystanders that do not bind to DNA and that stimulation causes neither the loss nor recruitment of HDAC-1 to the nuclear p65 pool (Figure 6). Nevertheless, these separate findings reinforce our hypothesis that HDACs regulate nuclear NF-κB activity.

An important aspect of our study is the provision of a mechanism for the role of p50 homodimers in regulating NF-κB. It is known that p50 homodimers exist in the nucleus of unstimulated cells (Kang et al., 1992; Ten et al., 1992), possibly because of the inability of IκBs to retain p50 in the cytosol. Thus, newly formed p50 homodimers translocate directly to the nucleus, associate with HDAC-1, and repress transcription from κB-dependent promoters. However, NF-κB heterodimers bind with greater affinity to most κB sites on natural promoters (Kunsch et al., 1992; Phelps et al., 2000), enabling signal-induced heterodimers to displace p50-HDAC-1 and activate transcription. This paradigm was elegantly demonstrated for the IFN-β promoter that is occupied by p50 in uninfected cells but becomes bound by p50:p65 after viral infection (Parekh and Maniatis, 1999; Senger et al., 2000). Consistent with our model of activated NF-κB recruiting CBP/p300 (Zhong et al., 1998), these groups also demonstrated that associated histones are acetylated upon infection. Such use of repressive forms of transcription factors in resting cells has been reported for steroid hormone receptors and may be true for other inducible transcription factors (Alland et al., 1997; Heinzel et al., 1997). However, our results provide compelling evidence for a regulatory mechanism involving phosphorylation to determine association of nuclear p65 with HDAC-1 or CBP/p300.

Our TSA studies strongly suggest that the activity of HDAC-1 maintains the transcriptional repression by p50-HDAC-1 complexes, as inhibition of HDAC activity upregulates a subset of κB-dependent genes. This is supported by our ChIP analysis demonstrating that p50 and HDAC-1 associate with the TSA-sensitive IL-6 promoter in resting cells. Moreover, TSA treatment caused acetylation of bound H4, and our studies with LPS demonstrated that a physiological NF-κB activator induced IL-6 promoter-associated H4 acetylation. The response to LPS was accompanied by no change in p50 association, an increase in p65, and loss of HDAC-1, supporting a model in which p50-HDAC-1 complexes are replaced by p50:p65 after stimulation. These data also support the notion that chromatin remodeling by recruited CBP/p300 activity counteracts the repression by p50-HDAC-1 and has a crucial role in κB-dependent gene expression. Intriguingly, the κB-dependent promoters of some constitutively or rapidly activated genes have high levels of associated H4-Ac in resting cells (Saccani et al., 2001), and we therefore speculate that such promoters are not bound by repressive p50-HDAC-1 complexes.

It is important to distinguish the effects of TSA on p50-HDAC-1 from those of deleting p50. Cells lacking p50 would not have p50:HDAC-1 bound to κB sites, yet gene expression need not occur, as transcription requires binding of other positive-acting factors. However, if some NF-κB-regulated promoters have distinct HDAC-independent transcription factors already bound, deletion of repressive p50:HDAC-1 may cause expression of these genes. In keeping with this model, TSA induced IL-6 and iNOS expression, whereas only iNOS

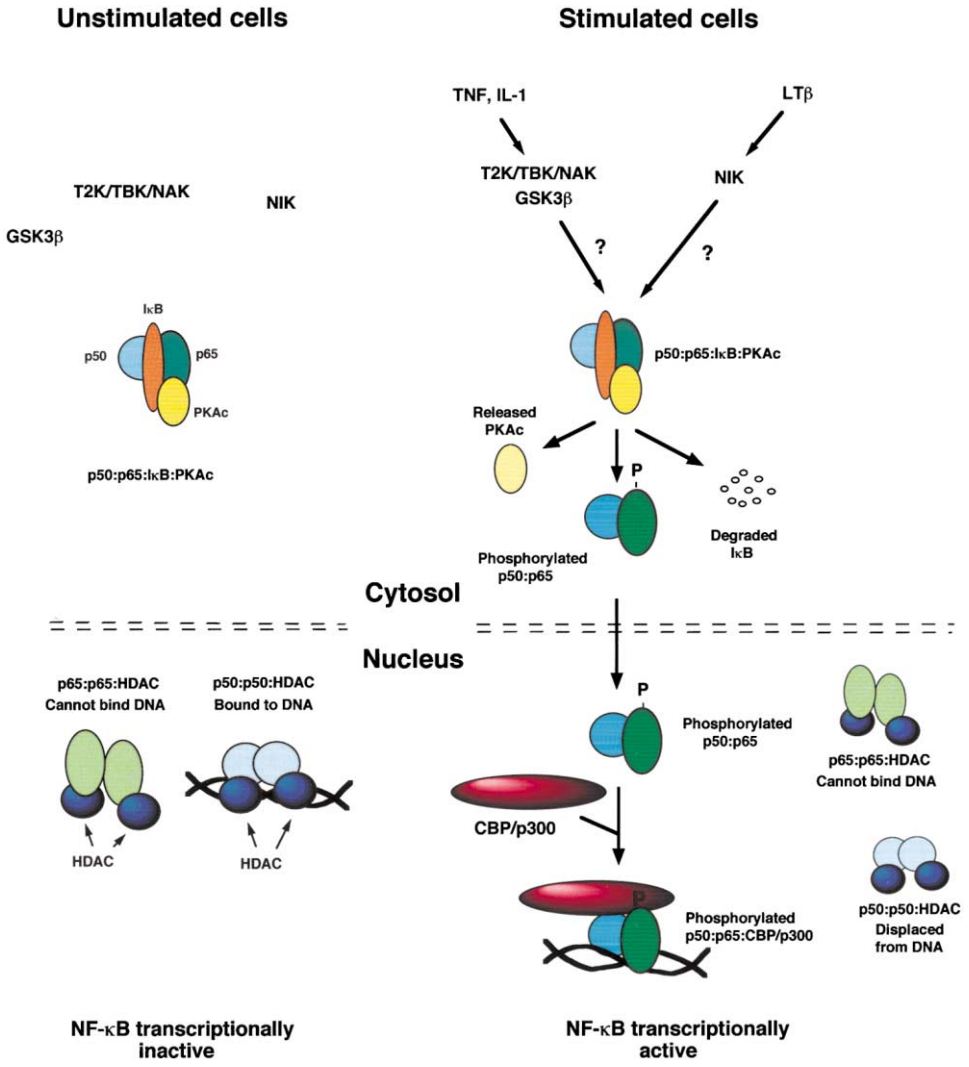

Figure 6. Phosphorylation Determines Whether Nuclear NF-κB Associates with Histone Acetylase or Deacetylase

In unstimulated cells (left), transcriptionally inactive nuclear NF-κB consists of p50 or p65 homodimers bound to HDAC-1, and while p50-HDAC-1 binds to DNA, unphosphorylated p65-HDAC-1 complexes do not. In contrast, signal-induced transcriptionally active NF-κB entering the nucleus (right) is phosphorylated and associated with CBP/p300 and can displace p50-HDAC-1 complexes from DNA. This mechanism insures that only signal-induced NF-κB drives NF-κB-dependent gene expression.

was increased in resting $p50^{-/-}$ cells. It therefore appears that p50 homodimers recruit HDAC-1 to inactivate the promoters of a defined subset of NF-κB-regulated genes.

The effects of HDAC-1 and CBP/p300 underscore the importance of acetylation in regulating NF-κB activity, although the identity of CBP/p300 targets remains to be fully determined. CBP/p300 can acetylate the four core histones, loosening chromatin and facilitating transcription (Struhl, 1998). In addition to our findings with IL-6, histones associated with other NF-κB-dependent genes are also acetylated following stimulation (El Kharroubi et al., 1998; Senger et al., 2000). Alternative targets include p53, where acetylation is important for CBP-mediated p53-dependent transcription, (Gu and Roeder, 1997) although unlike p53 and despite many attempts, we have not detected p65 acetylation by CBP/p300 (unpublished data). Intriguingly, a recent report demonstrated p300-dependent p65 acetylation that is reversed by HDAC-3 (Chen et al., 2001) and concluded that deacetylated p65 binds to IκBα and is exported from the nucleus causing downregulation of signal-induced NF-κB activity. However, the effect of HDAC-3 on acetylated histones associated with transcriptionally active p65-dependent genes was not examined, and the level of acetylated p65 was very low, suggesting that the full complement of nuclear p65 is not acetylated. Thus, it appears that the major effect of CBP/p300 on NF-κB-dependent transcription is via acetylation of histones or other proteins in the chromatin remodeling and transcriptional apparatus.

In summary, we have shown that p65 phosphorylation determines whether nuclear NF-κB associates with HDAC-1 (inactive) or CBP/p300 (active) and that p50-HDAC-1 represses NF-κB-dependent gene expression in resting cells. Such a regulatory mechanism (Figure

6) ensures that only stimulus-induced NF-κB activates transcription, and NF-κB in the nucleus for any other reason is transcriptionally silent. This mechanism is unique among the inducible transcription factors, as it imposes an additional layer of control on NF-κB that most likely reflects the necessity of maintaining it as a true inducible transcription factor.

Experimental Procedures

Reagents

HDAC-1 and CBP-HAT$^-$ expression vectors were gifts from Drs. S. Schreiber (Harvard University) (Taunton et al., 1996) and M. Rosenfeld (UCSD) (Korzus et al., 1998), respectively. Anti-p65 and anti-p50 were from Biomol (Plymouth Meeting, PA) or Santa Cruz (Santa Cruz, CA); one antibody against human HDAC-1 was from Dr. S. Schreiber and others were from Santa Cruz or Sigma (St. Louis, MO); anti-CBP and anti-HA were from Santa Cruz. Anti-p50 and anti-HDAC-1 (Santa Cruz), anti-p65 (Biomol), and anti-H4Ac (Upstate Biotechnologies, Lake Placid, NY) were used for ChIP. Protein G Sepaharose was from Amersham Pharmacia (Piscataway, NJ), TSA was from Biomol, and PMA, PHA, ionomycin, and LPS were all from Sigma.

Transient and Stable Transfections

For transient transfections, DNA was mixed with Fugene6 (Roche, Indianapolis, IN; 3:1 ratio) in serum-free medium and then incubated (15 min; 22°C) before adding to cells. Cells were lysed in passive lysis buffer (Promega, Madison, WI) and assayed (Kopp et al., 1999). HEK293 cell lines expressing pBIIx-luc or pFluc were made as described previously (Kopp et al., 1999). Cells were cotransfected with pCI-*neo* (10:1 ratio) using Fugene6 and selected with G418 (Life Technologies, Rockville, MD). Experiments were repeated using at least three separate lines.

Immunoprecipitation (IP) and Immunoblotting (IB)

All IP and IB analysis was performed as described by Zhong et al. (1997). Cells were lysed in buffer containing 200 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM DTT, and protease inhibitors (Roche).

In Vitro Interaction Assays

GST-p65 was generated and assays were performed as previously described (Zhong et al., 1997). For GST-p50 and GST-HDAC-1, cDNAs were cloned into pGEX vectors (Amersham Pharmacia), and proteins were purified using glutathione-agarose. Target proteins were made using the TNT-T7 Quick system (Promega).

EMSA

EMSAs were performed as described previously (Zhong et al., 1997). Samples contained either 2 μl of in vitro translated proteins or 10 μg of nuclear extracts, and for supershifts, 5μl of anti-HDAC-1 or 1μl of anti-p65 or anti-p50. Samples were incubated (1 hr; 4°C) and then electrophoresed and visualized by autoradiography.

RPA and RT-PCR

RPA was performed using cytokine templates (Pharmingen, San Diego, CA) following the manufacturer's protocol. For RT-PCR, RNA from thymocytes or splenocytes from B6 wild-type or p50$^{-/-}$ mice (Jackson Labs, Bar Harbor, ME) prepared using Trizol (GIBCO-BRL, Rockville, MD) was amplified using Superscript II and Taq polymerase (GIBCO-BRL). Primer sequences are available on request.

Chromatin Immunoprecipitation

Analysis was performed following a kit protocol (Upstate Biotechnology). Chromatin from 3×10^6 HEK293 or RAW 264.7 cells sheared using a Virtis (Gardiner, NY) Virsonic sonicator (4x10 s; 1/3 power) was precleared with salmon sperm DNA-saturated protein G Sepharose, then precipitated using specific antibodies or beads alone. Samples were analyzed by PCR (30–34 cycles) using HotStarTaq polymerase (Qiagen, Valencia, CA) and primers to amplify either the IL-6 κB site (TTGCGATGCTAAAGGACG; TGTGGAGAAGGAGTTCA TAGC) or a 3′ gene segment (GGTGACAGAACGAGACCTTG; TTG GGTCAGGGGTGGTTATTGC).

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References

Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R.A. (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature *387*, 49–55.

Ashburner, B.P., Westerheide, S.D., and Baldwin, A.S., Jr. (2001). The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol. Cell. Biol. *21*, 7065–7077.

Baer, M., Dillner, A., Schwartz, R.C., Sedon, C., Nedospasov, S., and Johnson, P.F. (1998). Tumor necrosis factor alpha transcription in macrophages is attenuated by an autocrine factor that preferentially induces NF-kappaB p50. Mol. Cell. Biol. *18*, 5678–5689.

Beg, A.A., Sha, W.C., Bronson, R.T., Ghosh, S., and Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature *376*, 167–170.

Bird, T.A., Schooley, K., Dower, S.K., Hagen, H., and Virca, G.D. (1997). Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. J. Biol. Chem. *272*, 32606–32612.

Bohuslav, J., Kravchenko, V.V., Parry, G.C., Erlich, J.H., Gerondakis, S., Mackman, N., and Ulevitch, R.J. (1998). Regulation of an essential innate immune response by the p50 subunit of NF-kappaB. J. Clin. Invest. *102*, 1645–1652.

Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W.J., et al. (2000). Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. EMBO J. *19*, 4976–4985.

Cha-Molstad, H., Agrawal, A., Zhang, D., Samols, D., and Kushner, I. (2000). The Rel family member p50 mediates cytokine-induced C-reactive protein expression by a novel mechanism. J. Immunol. *165*, 4592–4597.

Chen, L., Fischle, W., Verdin, E., and Greene, W.C. (2001). Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science *293*, 1653–1657.

Dechend, R., Hirano, F., Lehmann, K., Heissmeyer, V., Ansieau, S., Wulczyn, F.G., Scheidereit, C., and Leutz, A. (1999). The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators. Oncogene *18*, 3316–3323.

El Kharroubi, A., Piras, G., Zensen, R., and Martin, M.A. (1998). Transcriptional activation of the integrated chromatin-associated human immunodeficiency virus type 1 promoter. Mol. Cell. Biol. *18*, 2535–2544.

Fujita, T., Nolan, G.P., Ghosh, S., and Baltimore, D. (1992). Independent modes of transcriptional activation by the p50 and p65 subunits of NF-kappa B. Genes Dev. *6*, 775–787.

Fujita, T., Nolan, G.P., Liou, H.C., Scott, M.L., and Baltimore, D. (1993). The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. *7*, 1354–1363.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. *16*, 225–260.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. *14*, 121–141.

Gu, W., and Roeder, R.G. (1997). Activation of p53 sequence-specific

DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595–606.

Hassig, C.A., Fleischer, T.C., Billin, A.N., Schreiber, S.L., and Ayer, D.E. (1997). Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell *89*, 341–347.

Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., Brard, G., Ngo, S.D., Davie, J.R., et al. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature *387*, 43–48.

Heissmeyer, V., Krappmann, D., Wulczyn, F.G., and Scheidereit, C. (1999). NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. EMBO J. *18*, 4766–4778.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86–90.

Huang, T.T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000). A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes. Proc. Natl. Acad. Sci. USA *97*, 1014–1019.

Ito, K., Barnes, P.J., and Adcock, I.M. (2000). Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. Mol. Cell. Biol. *20*, 6891–6903.

Kang, S.M., Tran, A.C., Grilli, M., and Lenardo, M.J. (1992). NF-kappa B subunit regulation in nontransformed CD4+ T lymphocytes. Science *256*, 1452–1456.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-kappaB activity. Annu. Rev. Immunol. *18*, 621–663.

Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C.A., and Ghosh, S. (1999). ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. Genes Dev. *13*, 2059–2071.

Korzus, E., Torchia, J., Rose, D.W., Xu, L., Kurokawa, R., McInerney, E.M., Mullen, T.M., Glass, C.K., and Rosenfeld, M.G. (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science *279*, 703–707.

Kunsch, C., Ruben, S.M., and Rosen, C.A. (1992). Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. Mol. Cell. Biol. *12*, 4412–4421.

Laherty, C.D., Yang, W.M., Sun, J.M., Davie, J.R., Seto, E., and Eisenman, R.N. (1997). Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell *89*, 349–356.

Ledebur, H.C., and Parks, T.P. (1995). Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. Essential roles of a variant NF-kappa B site and p65 homodimers. J. Biol. Chem. *270*, 933–943.

Lee, S.K., Kim, J.H., Lee, Y.C., Cheong, J., and Lee, J.W. (2000). Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. J. Biol. Chem. *275*, 12470–12474.

Li, Q., Van Antwerp, D., Mercurio, F., Lee, K.F., and Verma, I.M. (1999). Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science *284*, 321–325.

Madrid, L.V., Mayo, M.W., Reuther, J.Y., and Baldwin, A.S., Jr. (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J. Biol. Chem. *276*, 18934–18940.

Nagy, L., Kao, H.Y., Chakravarti, D., Lin, R.J., Hassig, C.A., Ayer, D.E., Schreiber, S.L., and Evans, R.M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell *89*, 373–380.

Parekh, B.S., and Maniatis, T. (1999). Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. Mol. Cell *3*, 125–129.

Phelps, C.B., Sengchanthalangsy, L.L., Malek, S., and Ghosh, G. (2000). Mechanism of kappa B DNA binding by Rel/NF-kappa B dimers. J. Biol. Chem. *275*, 24392–24399.

Plaksin, D., Baeuerle, P.A., and Eisenbach, L. (1993). KBF1 (p50 NF-kappa B homodimer) acts as a repressor of H-2Kb gene expression in metastatic tumor cells. J. Exp. Med. *177*, 1651–1662.

Saccani, S., Pantano, S., and Natoli, G. (2001). Two waves of nuclear factor kappaB recruitment to target promoters. J. Exp. Med. *193*, 1351–1359.

Schmitz, M.L., and Baeuerle, P.A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. EMBO J. *10*, 3805–3817.

Senger, K., Merika, M., Agalioti, T., Yie, J., Escalante, C.R., Chen, G., Aggarwal, A.K., and Thanos, D. (2000). Gene repression by coactivator repulsion. Mol. Cell *6*, 931–937.

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). Transcriptional activation by NF-kappaB requires multiple coactivators. Mol. Cell. Biol. *19*, 6367–6378.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. *12*, 599–606.

Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408–411.

Ten, R.M., Paya, C.V., Israel, N., Le Bail, O., Mattei, M.G., Virelizier, J.L., Kourilsky, P., and Israel, A. (1992). The characterization of the promoter of the gene encoding the p50 subunit of NF-kappa B indicates that it participates in its own regulation. EMBO J. *11*, 195–203.

Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motoyama, N., Ikeda, K., et al. (2000). NAK is an IkappaB kinase-activating kinase. Nature *404*, 778–782.

Udalova, I.A., Richardson, A., Denys, A., Smith, C., Ackerman, H., Foxwell, B., and Kwiatkowski, D. (2000). Functional consequences of a polymorphism affecting NF-kappaB p50-p50 binding to the TNF promoter region. Mol. Cell. Biol. *20*, 9113–9119.

Wang, D., Westerheide, S.D., Hanson, J.L., and Baldwin, A.S., Jr. (2000). Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J. Biol. Chem. *275*, 32592–32597.

Yin, L., Wu, L., Wesche, H., Arthur, C.D., White, J.M., Goeddel, D.V., and Schreiber, R.D. (2001). Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in NIK-deficient mice. Science *291*, 2162–2165.

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. Cell *89*, 413–24.

Zhong, H., Voll, R.E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol. Cell *1*, 661–671.