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IκB Kinase α-Mediated Derepression of SMRT Potentiates Acetylation of RelA/p65 by p300

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Over the last several years, significant progress has been made in identifying chromatin-regulated events that govern NF-κB transcription. Using either laminin attachment or tumor necrosis factor alpha as a physiological stimulus of NF-κB activation, we demonstrate that IκB kinase α (IKKα) is recruited to chromatin in distinct phases. In the initial phase, IKKα is responsible for derepressing the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT)-histone deacetylase 3 (HDAC3) corepressor complex from the p50 homodimer. However, in the latter phase, chromatin-bound IKKα coordinates the simultaneous phosphorylation of RelA/p65(S536) and SMRT(S2410) as detected by chromatin immunoprecipitation (ChIP) assays. Although phosphorylated SMRT remains bound to the active p50-RelA/p65 heterodimer of NF-κB, derepression of SMRT is evidenced by the loss of chromatin-associated HDAC3 activity. ChIP and re-ChIP analysis demonstrates that phosphorylation of RelA/p65(S536) and SMRT(S2410) occurs prior to acetylation of RelA/p65 at K310. Moreover, IKKα-induced phosphorylation of RelA/p65(S536) displaces corepressor activity, allowing p300-mediated acetylation of RelA/p65. Introduction of nonphosphorylatable mutants of RelA/p65 and SMRT proteins or the inhibition of IKK activity results in active repression of NF-κB promoters by tethering the SMRT-HDAC3 complex. Similar to phosphorylation within the Rel homology domain of RelA/p65, which governs an exchange of HDAC1 for CBP/p300 acetyltransferases, we demonstrate that phosphorylation within the transactivation domain of RelA/p65(S536) displaces SMRT-HDAC3 repressor activity, allowing p300 to acetylate RelA/p65.

The transcription factor NF-κB plays an important role in many cellular processes, including inflammation, proliferation, and cell survival (8, 10, 32, 38). Members of the NF-κB family include RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (25). The Rel family members function as either homodimers or heterodimers with distinct specificity for *cis*-binding elements located within the promoter domains of NF-κB-regulated genes (44). Classical NF-κB, composed of the RelA/p65 and p50 heterodimer, is the best-studied form of NF-κB and most notable for its ability to upregulate genes that protect cells from apoptosis (10, 32). Prior to cellular stimulation, classical NF-κB resides in the cytoplasm as an inactive complex bound to the IκB inhibitor proteins. The IκB family of proteins includes IκBα, IκBβ, IκBε, IκBγ, p105, p100, and Bcl-3. Following cellular stimulation, a signal transduction cascade is activated that converges on the IκB kinase (IKK) signalosome complex (22, 25). The classical IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ/NEMO (NF-κB essential modulator). Once the IKK signalosome complex phosphorylates IκB, it is polyubiquitinated by an SCF-type E3 ligase, $E3RS^{I\kappa B/\beta\text{-}TrCP}$, and subsequently degraded by the 26S proteasome (22, 31). Degradation of IκB releases NF-κB, allowing the transcription factor to translocate to the nucleus to initiate transcription. While IKKβ and IKKγ activities are critical for IκB phosphorylation, IKKα is capable of phosphorylating acetyltransferases, including steroid receptor coactivator 3 (SRC-3) and the CREB-interacting binding protein (CBP) (66, 67). IKKα has also been shown to be a chromatin-associated kinase capable of contributing to NF-κB- and estrogen receptor-mediated transcription by phosphorylating histone H3 and the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) (4, 5, 26, 47, 67).

In unstimulated cells, NF-κB-regulated genes are basally repressed by p50 or p52 homodimers that tether corepressor complexes (40, 61). The p50 homodimer has been shown to recruit repressor complexes composed of SMRT and histone deacetylase 3 (HDAC3) or Bcl-3, nuclear corepressor (N-CoR), and HDAC3 (7, 26). Therefore, before transcription is initiated, the cell must first remove the repressor complexes from chromatin through a process called derepression (7). NF-κB-regulated genes that tether Bcl-3 become derepressed through mechanisms that involve MEKK1-dependent phosphorylation of TAB2, which facilitates the nuclear export of N-CoR and HDAC3 (7). Recently our laboratory demonstrated that classical NF-κB-regulated promoters become derepressed by recruiting chromatin-associated IKKα, which is responsible for stimulating nuclear export and degradation of SMRT through the recruitment of TBL1/TBLR1 and Ubc5 ubiquitin-dependent complex (26). This inability to derepress the corepressor complexes greatly impedes NF-κB transcription, suggesting that this is a critical prerequisite step in NF-κB transcriptional activation (7, 26, 48).

Classical NF-κB transcription is governed by the ability of RelA/p65 to recruit both histone acetyltransferase (HAT) activity and ATP-dependent remodeling complexes (40). RelA/p65 has been shown to recruit HAT-associated complexes con-

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taining p300, CBP, p300/CBP-associated factor, and SRC-1, -2, and -3 proteins (42, 59, 60, 65). The recruitment of HAT activity is governed by phosphorylation of RelA/p65 within the Rel homology domain (RHD) at S276 by the catalytic protein kinase A subunit or by mitogen and stress-activated protein kinase 1 and at S311 by the protein kinase Cζ (20, 63, 72). The transactivation potential and DNA binding activity of RelA/p65 are regulated by p300-induced acetylation (14, 16, 33, 42, 69, 72). p300-induced acetylation of RelA/p65 at K310 is associated with full NF-κB transcriptional activity, suggesting that acetylation within the RHD of RelA/p65 is required for NF-κB-mediated enhanceasome activity (16, 17, 69). Moreover, consistent with acetylation governing NF-κB transcription, RelA/p65 activity has been shown to be repressed by SMRT and N-CoR corepressor proteins as well a HDAC1, HDAC2, HDAC3, and the NAD-dependent deacetylase SIRT1 (6, 7, 14, 26, 35, 48, 69, 72).

RelA/p65 is also posttranslationally regulated by serine/threonine phosphorylation within the transactivation domains (TA1 and TA2) of the NF-κB subunit. The transactivation potential of RelA/p65 is repressed by phosphorylation at threonine 502 by $p14^{ARF}$ or by glycogen synthase kinase 3β-induced phosphorylation of RelA/p65 at S468 (11, 51, 52). In contrast, phosphorylation within the TA1 domain of RelA/p65 has been associated with enhanced transactivation function. Although the transactivation potential of RelA/p65 is upregulated by phosphorylation at S529 by casein kinase II (64), the most physiologically inducible phosphorylation site reported for RelA/p65 occurs at S536. Unlike other serine residues with the TA1 domain of RelA/p65, S536 is conserved across many different species (37). Numerous serine/threonine kinase cascades have been shown to phosphorylate RelA/p65(S536), including IKKα, IKKβ, IKKε, ribosomal S6 kinase 1, TRAF family member-associated-binding kinase 1, transforming growth factor beta-activated kinase 1, and NF-κB-activating kinase-associated protein 1 (9, 12, 28, 30, 37, 45, 46, 56, 57, 68). Although the phosphorylation of RelA/p65(S536) corresponds with increased transactivation potential of NF-κB, the role of this phosphorylation site remains elusive. In this report, we demonstrate for the first time that IKKα-induced phosphorylation of RelA/p65(S536) coincides with phosphorylated SMRT(S2410) and a loss of HDAC3 activity on the NF-κB-regulated promoters. IKKα is required to stimulate derepression of the SMRT-HDAC3 complex from the NF-κB heterodimer, allowing p300 to acetylate RelA/p65 at K310 for full transcriptional activity. Work presented here demonstrates the importance of IKKα-induced phosphorylation of RelA/p65 as a critical determinant for active derepression from the SMRT-HDAC3 complex.

MATERIALS AND METHODS

Cell culture, reagents, and plasmid constructs. HEK 293T and DU145 cell lines were obtained from ATCC. For attachment assays, cells were grown to 75% confluence, washed with phosphate-buffered saline (PBS), trypsinized with 1× trypsin, and replated in serum-free medium on laminin-coated plates for various time periods (Discovery Labware, catalog no. 354452). Bay 11-7082 was obtained from Calbiochem (catalog no. 196870). Recombinant tumor necrosis factor alpha (TNF-α) was obtained from Sigma. TNF-α was added to cells (10 ng/ml) which had been serum starved for 18 h prior to stimulation. Antibodies used in the studies include the following: M2 Flag and α-tubulin (Sigma F3165 and T9026), IKKα, IKK pS180/181, IKKβ, IKKγ, IκBα pS32, and RelA/p65 pS536 (2682, 2681, 2684, 2695, 9241, and 3031, respectively; Cell Signaling), p50/p105, HDAC1, HDAC3, IκBα, RNA polymerase II, normal immunoglobulin G, and hemagglutinin (HA) (SC-114, SC-7872, SC-8138, SC-371, SC-9001, SC-2027, and SC-805; Santa Cruz), RelA/p65, histone H3, and p300 (06-418, 06-599, and 05-267; Upstate), and SMRT (PA1-842; ABR). The phospho-specific anti-SMRT (pS2410) and the anti-acetyl RelA/p65 (AcK310) antibodies were developed in collaboration with Upstate. Plasmids encoding the Gal4 luciferase reporter and Gal4-p65 were described previously (58). The full-length pGEM-p65 plasmid used in in vitro transcription and translation was described previously (6). Expression plasmids encoding the glutathione *S*-transferase (GST)-p65(354-551) protein were previously described (56). Site-directed mutagenesis (S→A) was performed on pSP72-SMRT (43), Flag-SMRT, HA-RelA/p65, and GST-p65 plasmids with the Stratagene mutagenesis kit (catalog no. 200514). All mutant plasmids were confirmed by DNA sequencing.

Transfections, siRNA silencing, luciferase assays, and Western blotting. Unless otherwise indicated, DU145 and HEK 293T cells were transfected with the indicated plasmids (2 μg each) in the presence of Polyfect transfection reagent (catalog no. 3001105; QIAGEN). For typical Western blot analysis of ectopically expressed transgenes, cells were harvested (24 h posttransfection), lysed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes using the NOVEX (Invitrogen) system. All primary antibodies were incubated overnight at 4°C. The next day, membranes were washed and incubated for 1 h at room temperature with the appropriate secondary antibody. Chemoluminescence was performed with an Amersham ECL detection reagent (catalog no. RPN 2132). Alternatively, Western blot analysis for the detection of chromatin-associated proteins was performed as follows. Cells were transfected with either small interfering RNA (siRNA) control or IKKα (200 nM each; D001206-13 and M003473; Dharmacon), as specified by the manufacturer, using oligofectamine (catalog no. 12252-011; Invitrogen). Twenty-four hours later, cells were transfected with expression vectors encoding Flag-RelA/p65 using Polyfect. Cells were harvested 48 h later and plated onto laminin-coated plates for 30 min. Cells were harvested, and soluble proteins were isolated in radioimmure precipitation (RIPA) assay buffer. Chromatin-associated proteins were retrieved from cellular pellets using 2% SDS. Protein expression and chromatin localization were analyzed by Western blot analysis as described above. All transfections used to analyze ectopic expression of transgene products by chromatin immunoprecipitation (ChIP) were conducted with reduced amounts of expression vectors. Briefly, HEK 293T cells were cotransfected with expression vectors (200 ng [each] per 100-mm dish) in a total of 8 μg using Polyfect. These plasmid amounts were determined to be optimum for RelA/p65 expression because they allowed detection of ectopic chromatin-associated expression following stimulation without gross overexpression. Luciferase assays were performed as follows. DU145 cells were transfected with the indicated plasmids for 24 h, harvested, and lysed with Promega 5× reporter lysis buffer. Lysed cell extract (50 μg) was then added with Luciferase assay reagent and quantified using a LMax II 384 Molecular Devices luminometer. All luciferase values were normalized to total protein. Transfection data are the means ± standard deviations for three independent experiments performed in triplicate.

In vitro association and in vitro kinase interaction assays. In vitro association assays were performed as described previously (6). Briefly, the full-length RelA/p65 protein (1 to 551) was in vitro transcribed and translated in the presence of [^{35}S]methionine (NEN) using the TNT T7/SP6 coupled reticulocyte lysate system according to the manufacturer's specifications (catalog no. L5020; Promega). One-tenth of the ^{35}S-labeled RelA/p65 was mixed with GST protein or various GST-SMRT proteins in binding buffer (PBS with 1% Triton X-100) in the presence of glutathione-agarose beads (Sigma). Reactions were rocked at 4°C for 1 h. Glutathione beads were washed three times with 1× PBS containing 1% Triton X-100. Samples were resuspended in loading buffer and analyzed by SDS-PAGE. The gels were dried and visualized by autoradiography. ^{35}S-radiolabeled RelA/p65 protein was loaded on the polyacrylamide gel for input control. In vitro kinase interaction assays were performed as described previously (72). Briefly, GST or GST-p65 proteins (5 μg) were incubated in the presence or absence of 1 μg recombinant IKKα (catalog no. 14-461; Upstate) for 30 min at 30°C in the presence of kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μm ATP). Alternatively, kinase reactions were carried out on in vitro-transcribed and -translated ^{35}S-labeled SMRT proteins. IKK activity was inhibited in all reactions by the addition of 0.5 M EDTA. GST or GST-p65 proteins were mixed with ^{35}S-labeled SMRT protein in binding buffer and incubated at 4°C for 1 h with gentle tumbling. Samples were washed, loaded, and resolved by SDS-PAGE. Dried gels were analyzed by autoradiography as described above. For control purposes, IKKα kinase reactions were carried out on GST, GST-p65, and SMRT proteins. Proteins were immu-

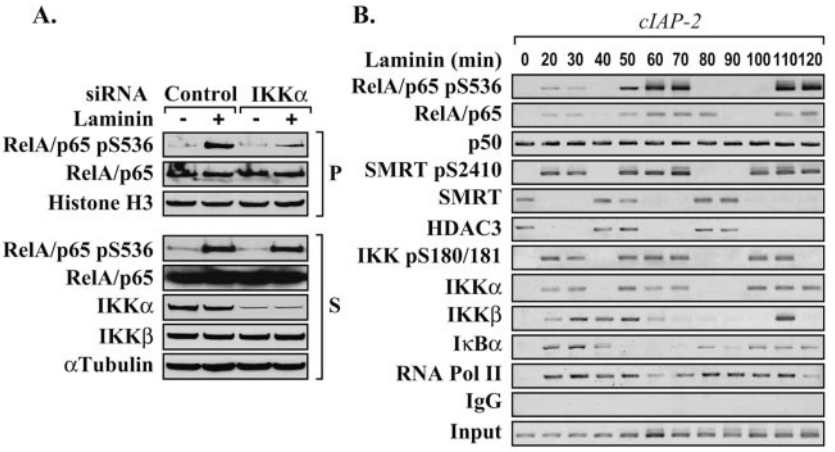

FIG. 1. Chromatin-associated IKKα activity corresponds with the detection of phosphorylated RelA/p65(S536) and SMRT(S2410) proteins. (A) Chromatin-associated RelA/p65 is phosphorylated at S536 in an IKKα-dependent manner following attachment to laminin. HEK 293T cells, transiently transfected with expression plasmid encoding Flag-RelA/p65, were treated with either control or IKKα siRNAs (200 nM, each) for 72 h before being replated on laminin for 30 min. Cellular proteins were isolated in RIPA buffer representing the soluble (S) fraction. Alternatively, cellular pellets (P), which constitute the chromatin-associated proteins, were resuspended in 2% SDS for isolation. Western blot analysis was performed using proteins isolated from both the S and P fractions. Ectopically expressed RelA/p65 was detected in the S and P fractions using Flag-specific antibody. Detection of histone H3 or α-tubulin served as a loading control for the protein fractions. (B) ChIP analysis of the *cIAP-2* promoter displays triphasic correlation between chromatin-bound IKKα activity and phosphorylated RelA/p65(S536) and SMRT(S2410) following attachment of DU145 cells to laminin. Data shown constitute a representative ChIP analysis of two individual experiments.

noblotted and analyzed for the presence of phosphorylated proteins using either anti-RelA/p65 pS536 or anti-SMRT p2410 antibody.

ChIP and re-ChIP assays. ChIP primers for the *cIAP-2* and *IL-8* promoters were described previously (26, 39, 69). For ChIP analysis involving ectopic expression of wild-type and mutant HA-RelA/p65 and wild-type and mutant Flag SMRT, HEK 293T cells were cotransfected with one-tenth of the normal level of expression plasmids (see above for details). Re-ChIP assays of ectopically expressed and endogenous proteins were performed as described previously (41). Briefly, single ChIP complexes were eluted by incubation for 30 min at 37°C in 25 μl 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1]) and subjected to another round of immunoprecipitation. ChIP analysis was carried out as described previously (26, 39, 69).

Quantitative real-time PCR and apoptosis assays. Total RNA, 50 ng, was converted to cDNA with oligo-dT primers using the Omniscript reverse transcriptase protocol (catalog no. 205110; QIAGEN). Each cDNA reaction mixture (5-μl aliquot) was used in 50 μl real-time PCR with TaqMan Universal PCR 2× master mix (catalog no. 4304437) supplemented with 200 μmol/liter (each) primer and 100 μmol/liter probe. Reactions were subjected to the following amplification conditions: 95°C for 900 s followed by 40 cycles of 95°C for 15 s and 60°C for 60 s using the 7500 Real-Time PCR system (PE Biosciences). The degree of change in transcripts was calculated as described previously (3). Briefly, we divided the ratio of the normalized copies of the experimental gene in dimethyl sulfoxide (DMSO) by the normalized copies of the experimental gene in Bay 11. The equation $Nc = (1 + E)\Delta C_T$ was used, where Nc = normalized copies of experimental gene per copy of the internal control, β-*glucuronidase* (*GUS*) housekeeping gene, E is the efficiency of the PCR, and the ΔC_T is the difference in the cycle threshold for the experimental gene and *GUS*. The PCR efficiency is determined by the difference between the slope of a standard curve generated for each gene (C_T versus log [total RNA]) from the expected slope in an ideal PCR. All runs were performed in duplicate. The sequences (5′ to 3′) for each primer and probe (Synthegen, Houston, TX) are as follows: *cIAP-2* forward, TCC GTC AAG TTC AAG CCA GTT; *cIAP-2* reverse, TCT CCT GGG CTG TCT GAT GTG; and *cIAP-2* probe, CCC TCA TCT ACT TGA ACA GCT GCT AT; *IL-8* forward primer, GTT TTT GAA GAG GGC TGA GAA TTC; *IL-8* reverse primer, CAT GAA GTG TTG AAG TAG ATT TGC TTG; and *IL-8* probe, ATC CAA GAA TCA GTG AAG ATG CCA GTG AAA CT; *GUS* forward primer, GAA AAT ATG TGG TTG GAG AGC TCA TT; *GUS* reverse primer, CCG AGT GAA GAT CCC CTT TTT A; and *GUS* probe, CCA GCA CTC TCG TCG GTG ACT GAC TGT TCA. Apoptosis was determined using the Cell Death Detection ELISA Plus kit (Roche, catalog no. 1585045) according to the manufacturer's instructions. All data were normalized to absorbance units per μg of protein.

RESULTS

IKKα activity corresponds with RelA/p65 and SMRT phosphorylation on chromatin. Using cell attachment to the extracellular matrix laminin as a physiological stimulus, we demonstrated previously a requirement for chromatin-associated IKKα to phosphorylate the corepressor SMRT for derepression of NF-κB transcription (26). Since both RelA/p65 and SMRT have been shown to be phosphorylated by IKKα (26, 30, 34, 46), we wanted first to determine whether there is a correlation between IKKα-induced phosphorylation of chromatin-associated RelA/p65 in our cell model system. To address this issue, we used IKKα siRNA knockdown to analyze the requirement of IKKα to phosphorylate RelA/p65 at S536 following attachment to laminin. To analyze chromatin-associated effects, we compared the protein phosphorylation status between soluble (S) and pellet (P) fractions. IKKα is chromatin associated, unlike other kinases capable of phosphorylating RelA/p65(S536) (4, 5, 12, 26, 34, 57, 67, 68). Therefore, we hypothesized that we could use the P fraction as a means to enrich IKKα-mediated phosphorylation of RelA/p65(S536). As shown in Fig. 1A, treatment of HEK 293T cells with IKKα siRNA significantly knocked down IKKα protein expression, compared to results for control siRNA-treated cells. As predicted, siRNAs to IKKα did not affect IKKβ, histone H3, or α-tubulin protein expression. Attachment of HEK 293T cells to laminin stimulated RelA/p65(S536) phosphorylation in siRNA control cells. However, siRNA knockdown of IKKα significantly diminished detection of RelA/p65(S536) phosphorylation in the chromatin-associated P fraction. As expected, the knockdown of IKKα did not significantly diminish

RelA/p65(S536) phosphorylation in the S fraction, presumably because there are several cytoplasmically localized kinases, in addition to IKKα, capable of phosphorylating RelA/p65(S536) (Fig. 1A). Unstimulated cells displayed low levels of phosphorylated RelA/p65(S536); however, phosphorylation was greatly potentiated following attachment to laminin. These results have led us to conclude that IKKα is required to phosphorylate chromatin-bound RelA/p65(S536).

To determine if there is a correlation between chromatin-bound IKKα activity and phosphorylation of RelA/p65 and SMRT at the endogenous level, we performed ChIP assays and evaluated chromatin-associated interactions over an extended-time-frame attachment to laminin. As shown in Fig. 1B, RelA/p65 was recruited to the *cIAP-2* promoter in a triphasic manner across several time frames (20 to 30, 50 to 80, and 110 to 120 min) in DU145 cells. In a similar manner, phosphorylated RelA/p65(S536) was found to correspond with the recruitment of RelA/p65 to chromatin in a triphasic manner following stimulation. As expected, the p50 DNA binding subunit of NF-κB did not show differences in *cIAP-2* promoter occupancy, since this component of NF-κB remained chromatin bound either as a p50 homodimer or as a p50-RelA/p65 heterodimer. Interestingly, we detected phosphorylated SMRT(S2410) across the same time frames as phosphorylated RelA/p65(S536). Moreover, SMRT phosphorylation at S2410 inversely correlated with total SMRT and HDAC3 occupancy on the *cIAP-2* promoter. To determine if RelA/p65 phosphorylation at S536 and SMRT phosphorylation at S2410 corresponded with IKK activity, additional ChIP assays were performed using the anti-IKK pS180/181 antibody, which recognizes chromatin-associated IKKα activity (26). Phosphorylated IKK activity was detected on the *cIAP-2* promoter over the same time frames as phosphorylated RelA/p65 and SMRT. Moreover, detection of activated IKK pS180/181 corresponded with IKKα promoter occupancy, while IKKβ failed to show chromatin interaction patterns that fully overlapped with RelA/p65 or SMRT phosphorylation. Although IκBα has been proposed to downregulate NF-κB and Notch-responsive gene targets through chromatin-mediated mechanisms (2, 14), the chromatin-associated IκBα protein did not correspond with SMRT-HDAC3-mediated repression in our model system. Modulation of phosphorylated RelA/p65 and SMRT proteins corresponded with the recruitment of RNA polymerase II, which, unlike NF-κB, displayed biphasic recruitment to the *cIAP-2* promoter.

It is intriguing that we were able to detect phosphorylated SMRT on chromatin using the phospho-specific anti-SMRT p2410 antibody, but were unable to detect SMRT using a pan anti-SMRT antibody that recognizes a centrally localized region of the SMRT protein (1302 to 1495). Since we already know that phosphorylation of SMRT(S2410) by IKKα stimulates nuclear export (26), it is likely that a portion of the chromatin-bound SMRT is stimulated for export during the initial derepression phase. However, it is likely that the pan anti-SMRT antibody is no longer able to recognize phosphorylated SMRT when bound to RelA/p65. However, SMRT derepression has occurred, as evidenced by the loss of chromatin-associated HDAC3 activity. Further evidence of derepression is provided by re-ChIP analysis (see Fig. 4C.) Regardless of the mechanism, what is important is that during initial SMRT derepression we observed a displacement of p50-p50 homodimers for the p50-RelA/p65 heterodimer. This process occurred concomitantly with recruitment of IKKα activity, the presence of phosphorylated RelA/p65 and SMRT proteins, and a loss of HDAC3 deacetylase activity.

IKKα regulates protein-protein interactions between RelA/p65 and SMRT in vitro. To determine which regions of SMRT interact with RelA/p65, we performed in vitro interaction assays by mixing in vitro-transcribed and -translated ^{35}S-labeled RelA/p65 protein with various GST-SMRT proteins. Consistent with a previous report (35), the RelA/p65 protein interacted with the SMRT corepressor protein within protein segments (2321 to 2525) that contained the receptor-interacting domains (RD). RelA/p65 interacted with SMRT in an additional region within the repression domain (1031 to 1596). We chose to focus on the RD I/II region of SMRT, since this domain of the protein has been shown to be required for SMRT-mediated repression of RelA/p65 transactivation potential and because the RD I region contains the IKKα phosphorylation residue (pS2410), which is critical for IKKα-mediated derepression (26, 35).

To better understand the significance of IKKα-induced phosphorylation of RelA/p65 and SMRT, we performed in vitro kinase interaction assays to elucidate whether phosphorylation of RelA/p65 and/or SMRT governs protein-protein interactions between these two molecules. In vitro kinase interaction assays have been used successfully to demonstrate that cPKA-induced phosphorylation of RelA/p65 at S276 is responsible for displacing HDAC1 interaction with RelA/p65 (72). As shown in Fig. 2B, irregardless of the reaction order, recombinant IKKα protein was able to displace ^{35}S-labeled SMRT (2004–2525) from GST-p65(354–551) protein (Fig. 2B). IKKα kinase effectively phosphorylated either GST-p65 or SMRT protein, as detected by immunoblot analysis using phospho-specific antibodies.

To demonstrate that the effects shown in Fig. 2B were due to IKKα-induced phosphorylation and not due to steric hindrance by the kinase or by other nonspecific mechanisms, several approaches were taken. First, we repeated in vitro kinase interaction assays using the protein phosphatase 2B (PP2B), and second, we employed site-directed mutants lacking the IKKα phosphorylation sites in GST-p65(S536A) and/or SMRT(S2028,2410A). As shown in Fig. 2C, the addition of PP2B at the end of the in vitro kinase reaction abolished IKKα-mediated phosphorylation, allowing GST-p65 interaction with SMRT. However, the addition of IKKα could not disrupt the protein-protein interaction between mutant GST-p65(S536A) and SMRT (Fig. 2C). As predicted, IKKα was able to phosphorylate SMRT and wild-type GST-p65 but not mutant GST-p65(S536A), as detected using phospho-specific antibodies. To understand if IKKα-induced phosphorylation was governing protein-protein interactions in both a RelA/p65- and SMRT-dependent manner, assays were repeated using GST-p65(S536A) and SMRT(S2028,2410A) mutants. In vitro kinase interaction assays show that IKK-induced phosphorylation of either GST-p65 or SMRT contributes equally to protein-protein interaction between these two molecules. Moreover, assays performed with site-directed mutants lacking both the IKKα phosphorylation sites in GST-p65(S536A) and those in SMRT(S2028,2410A) did not further enhance interaction in

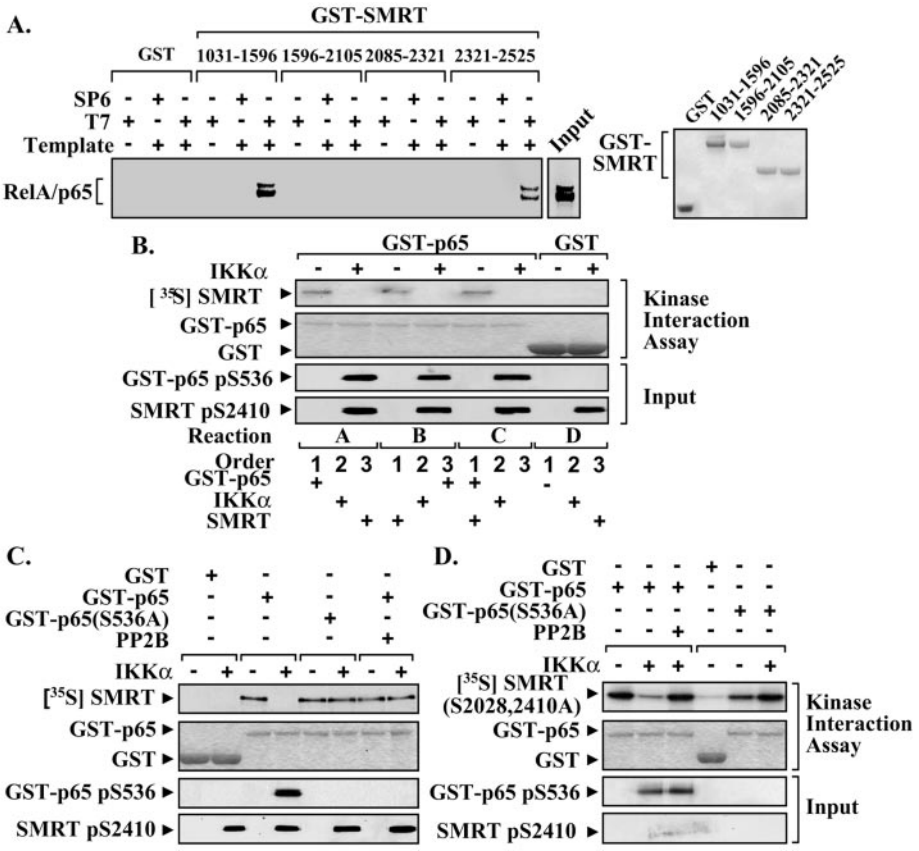

FIG. 2. IKKα phosphorylation mediates the disassociation between RelA/p65 and SMRT in vitro. (A) In vitro interaction assays display physical interaction between in vitro-transcribed and -translated ^{35}S-labeled RelA/p65 and GST-SMRT across two regions of the corepressor protein. RelA/p65 was transcribed in vitro using the T7 polymerase and translated using the TNT rabbit reticulocyte lysate system (Promega) in the presence of ^{35}S-labeled methionine. For control purposes, similar reactions were carried out either in the absence of the pGEM-p65 template or using the SP6 polymerase. Radiolabeled RelA/p65 proteins were mixed with GST-SMRT proteins, protein complexes isolated on glutathione-bound Sepharose beads and washed, and proteins were resolved by SDS-PAGE analysis. Dried gels were exposed to autoradiography. The mobility of ^{35}S-labeled T7-RelA/p65 input is shown. The Coomassie-stained gel demonstrates the relative levels of GST and GST-SMRT proteins used in the in vitro interaction assays. (B) In vitro kinase interaction assays demonstrate that the protein-protein interaction between RelA/p65 and SMRT is dissociated by IKKα phosphorylation. GST-RelA/p65(354–551) and ^{35}S-labeled SMRT(2004–2525) were incubated in the presence or absence of a recombinant IKKα enzyme (Upstate Biotechnology) combined in various reaction orders (first, second, third). Reaction orders are indicated with a plus sign. For example, in reaction A, GST-p65 was incubated with IKKα for 30 min, IKK activity was inhibited with 0.5 M EDTA, and GST-p65 proteins were washed and purified prior to the addition of ^{35}S-labeled SMRT for an additional 30 min. Complexes were washed, and GST-p65 proteins were purified; SDS dried gels were exposed to autoradiography. For in vitro interaction assays described for panels B, C, and D, input GST and GST-p65 proteins are shown for loading controls. To confirm that IKKα effectively phosphorylated GST-p65 and SMRT proteins in in vitro kinase reactions, Western blot analysis was performed on the inputs using α-RelA/p65(S536) or α-SMRT(S2410) antibodies. (C and D) IKKα phosphorylation disrupts SMRT and RelA/p65 protein association. In vitro interaction assays were performed as described for A in either the absence or presence of the PP2B phosphatase, using wild-type or mutant GST-p65 and SMRT proteins. The PP2B phosphatase enzyme was added 30 min before performing the binding assay with ^{35}S-labeled SMRT.

the in vitro kinase interaction assay (Fig. 2D). For control purposes, IKKα was found to effectively phosphorylate wild-type GST-p65 but not mutant SMRT(S2028,2410A). In vitro interaction assays shown in Fig. 2 indicate that SMRT interacts with RelA/p65, in part, through the RD I/II region. Moreover, IKKα-induced phosphorylation of GST-p65(354–551) at S536 or SMRT(2004–2525) at S2028 and S2410 displaces the interaction of these two proteins.

IKKα-induced phosphorylation of RelA/p65 and/or SMRT disrupts chromatin-associated repression complexes to potentiate NF-κB transcription. To elucidate the importance of IKKα-induced phosphorylation of RelA/p65(S536) and SMRT(S2410) for the transactivation potential of RelA/p65, transient transfections were performed using expression plasmids encoding wild-type or mutant SMRT or Gal4-p65 fusion proteins. The use of the Gal4-p65 system encoding amino acids 286 to 551 of RelA/p65 allows analysis of the importance of IKKα-mediated phosphorylation for RelA/p65 transactivation function without having to address additional complications of nuclear translocation. As demonstrated previously (26, 35), the transactivation potential of RelA/p65 is inhibited in cells expressing the SMRT corepressor, and yet the ability of SMRT to inhibit RelA/p65 activity is rescued by the expression of the IKKα protein (Fig. 3A). Surprisingly, IKKα is able to relieve SMRT-mediated repression of the Gal4-p65 protein despite the expression of the mutant SMRT(S2028,2410A) protein.

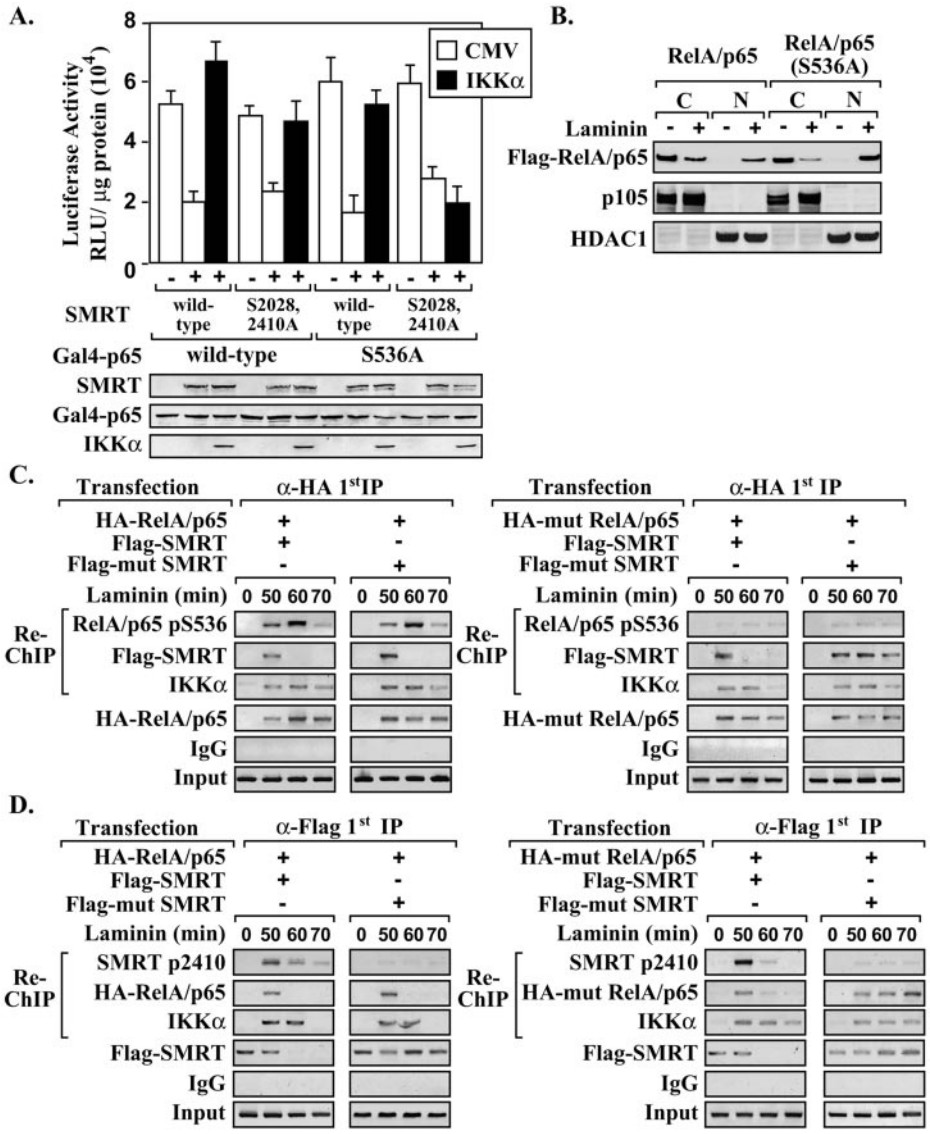

FIG. 3. IKKα-induced phosphorylation of either RelA/p65 or SMRT resulted in SMRT derepression. (A) DU145 cells were transfected with 4× Gal4 luciferase reporter and either wild-type Gal4-p65(286-551) or mutant Gal4-p65(286-551,S536A) fusion proteins. Additionally, cells were transfected with expression plasmids encoding wild-type SMRT, mutant SMRT(S2028,2410A), IKKα, and/or empty vector control (CMV). Luciferase activities were determined 24 h following transfection. Data represent the means ± standard deviations for three individual experiments performed in triplicate. Western blot analysis shows the relative expression of Gal4-p65, SMRT, and IKKα proteins. (B) Transient expression of wild-type RelA/p65 or mutant RelA/p65(S536A) displays similar patterns of nuclear translocation following attachment of HEK 293T cells to laminin for 30 min. Transfections described for panels B, C, and D were carried out using reduced levels (200 ng/100-mm dish) of expression plasmids encoding either wild-type or mutant RelA/p65 or SMRT as described in Materials and Methods. Cytoplasmic (C) and nuclear (N) extracts were isolated from transiently transfected HEK 293T cells expressing either Flag-tagged wild-type RelA/p65 or mutant RelA/p65(S536A) protein, and Western blot analysis was performed. HDAC1 and p105 served as nuclear and cytoplasmic loading controls. (C) IKKα-induced derepression is associated with phosphorylation of RelA/p65(S536) and SMRT(S2028,2410). HEK 293T cells were transfected as described for panel B using reduced amounts of plasmids encoding HA-tagged RelA/p65, mutant RelA/p65(S536A), Flag-tagged SMRT, and mutant SMRT(S2028,2410A) in various combinations. Thirty-six hours following transfection, cells were harvested and replated on laminin over the indicated time frames. ChIP and re-ChIP analyses were performed using primer sequences that correspond to the *cIAP-2* promoter. All assays shown in C were performed using anti-HA (α-HA) as the first immunoprecipitating (1st IP) antibody. Anti-HA antibody was used to pull down either RelA/p65 (left panel) or mutant RelA/p65 (right panel) protein-associated complexes. Secondary antibodies used in the re-ChIP analysis are shown. For control purposes, ChIP analysis was performed using anti-HA antibodies, demonstrating that the HA-tagged RelA/p65 and mutant RelA/p65 proteins became chromatin associated following stimulation. (D) Re-ChIP analysis on ectopically expressed SMRT demonstrates the importance of IKKα-induced phosphorylation of SMRT(S2028,2410) and RelA/p65(S536) proteins. Experiments in panel D were performed as described for panel C except that anti-Flag (α-Flag) was the first antibody used to immunoprecipitate wild-type or mutant SMRT protein.

The transactivation potential of RelA/p65 was unaltered in cells expressing the mutant Gal4-p65(S536A) protein, and yet IKKα was still able to rescue SMRT-mediated repression. However, IKKα was no longer able to rescue SMRT-mediated repression following expression of both mutant Gal4-p65(S536A) and SMRT(S2028,2410A). Differences in Gal4-p65-mediated luciferase activity were not due to differences in transgene expression, since similar levels of Gal4-p65, SMRT, and IKKα proteins were detected in transiently transfected cells (Fig. 3A). Results obtained using transfection assays suggest that there is cooperative binding between RelA/p65 and SMRT and that IKK-mediated phosphorylation of either protein governs derepression, allowing NF-κB transcription to occur.

To investigate the importance of IKK-induced phosphorylation of RelA/p65 and SMRT on chromatin dynamics, we analyzed the changes in chromatin occupancy in response to laminin attachment. Since transiently transfected plasmids encoding the Flag-RelA/p65 protein can easily overcome endogenous IκB expression when grossly overexpressed in HEK 293T cells, we titrated down on the amount of expression plasmid used in experiments described in Fig. 3B, C, and D. This approach also overcame our ability to detect constitutive RelA/p65(S536) phosphorylation in the absence of stimulation, which was observed with Fig. 1A. Before beginning experiments, we first analyzed whether the mutant RelA/p65(S536A) demonstrated differences in cytosolic and nuclear localization following stimulation. In contrast to previous reports (37), in which phosphorylation of RelA/p65(S536) contributes to nuclear export of NF-κB, we failed to observe differences in cytoplasmic or nuclear localization of the mutant RelA/p65 protein compared to the wild-type RelA/p65 protein in our model system (Fig. 3B). Detection of p105 and HDAC1 by Western blot analysis confirms the integrity of the cytoplasmic and nuclear extracts.

To determine the importance of IKKα-induced phosphorylation, we transfected HEK 293T cells with expression vectors encoding the wild-type RelA/p65, SMRT, or mutant RelA/p65(S536A) and SMRT(S2028,2410A) proteins and performed ChIP and re-ChIP analysis across the *cIAP-2* promoter following attachment of cells to laminin. Re-ChIP analysis allowed us to selectively analyze ectopically expressed RelA/p65 and SMRT proteins to determine how IKK-mediated phosphorylation of these two molecules governed protein-protein interaction on chromatin. We chose to evaluate the latter phase of RelA/p65(S536) recruitment to the *cIAP-2* promoter (50 to 70 min post-laminin attachment [T50 to T70]), since endogenous IKKα-mediated regulation was maximal during this time frame (Fig. 1B). As shown in Fig. 3C, expression of wild-type RelA/p65 but not the mutant RelA/p65(S536A) displayed phosphorylated RelA/p65 at 50 to 70 min post-laminin attachment. Ectopically expressed wild-type SMRT was observed on the *cIAP-2* promoter at 50 min in cells coexpressing either wild-type RelA/p65 or mutant RelA/p65. SMRT was not detected when replating on laminin was done (T0), presumably because RelA/p65 was not constitutively chromatin associated in unstimulated cells. This assumption is supported by the fact that ChIP analysis for Flag-SMRT identified the corepressor at T0 during times of basal repression (Fig. 3D). Consistent with data in Fig. 3A, mutant SMRT protein was still displaced from the *cIAP-2* promoter in cells coexpressing wild-type RelA/p65. However, it was not until both mutant SMRT and mutant RelA/p65 were coexpressed that SMRT remained associated with the *cIAP-2* promoter (Fig. 3C, right panel). IKKα but not IKKβ associated with RelA/p65 irregardless of whether the cells expressed the wild-type or mutant RelA/p65 and SMRT proteins (Fig. 3C and D). The importance of IKKα-induced phosphorylation of both RelA/p65 and SMRT is further supported by the fact that similar results were obtained in re-ChIP analysis when ectopically expressed SMRT was first immunoprecipitated and chromatin-associated protein-protein interactions were analyzed across the *cIAP-2* promoter (Fig. 3D). The discrepancies between in vitro kinase interaction assays and data presented in Fig. 3 are most likely due to the fact that the in vitro assays measured protein interactions between a portion of RelA/p65(354-551) and SMRT(2004-2525). However, when the full-length proteins are expressed and analyzed by ChIP analysis, our data indicate that IKKα is a powerful modulator of SMRT-mediated repression and that phosphorylation of either RelA/p65(S536) or SMRT(S2028,2410) disrupts corepressor activity.

Phosphorylation of RelA/p65 and SMRT by IKKα corresponds with a loss of SMRT-HDAC3 activity and p300-associated RelA/p65 acetylation. Although RelA/p65 has been shown to be acetylated at multiple lysine residues (16, 33), p300-induced acetylation of RelA/p65 is critical for NF-κB-mediated transcription (16, 17, 69). Based on the importance of K310 for transcriptional activity of NF-κB, we developed an antibody that specifically recognizes acetylated RelA/p65(K310) (Fig. 4A). To confirm the importance of IKKα-induced phosphorylation of RelA/p65, we repeated ChIP analysis to determine whether there is a correlation between RelA/p65 phosphorylation and p300-mediated acetylation of RelA/p65(K310). As shown in Fig. 4B, RelA/p65 was recruited to the *cIAP-2* promoter in a triphasic manner with kinetics similar to those shown in Fig. 1B. Importantly, we found that phosphorylated RelA/p65(S536) was detected on the *cIAP-2* promoter over the same time frame as was acetylated RelA/p65(K310). Our ability to detect RelA/p65(S536) corresponded with a recruitment of IKKα; however, p300 recruitment to the *cIAP-2* promoter displayed a biphasic pattern of chromatin association similar to that observed for RNA polymerase II (Fig. 1B).

To understand better the importance of IKKα-induced phosphorylation of RelA/p65 and to determine how this regulation governs RelA/p65 acetylation, we performed re-ChIP analysis, evaluating the regulation of endogenously expressed RelA/p65 and SMRT proteins on the *cIAP-2* and *IL-8* promoters. Results shown in Fig. 4B suggest that IKKα is capable of disrupting the ability of the SMRT-HDAC3 complex to repress RelA/p65 by IKKα phosphorylation of both RelA/p65 and SMRT. If this is correct, one could postulate that disruption of SMRT corepressor activity by IKKα contributes subsequently to the transactivation potential of RelA/p65 by promoting p300-induced RelA/p65(K310) acetylation. As shown in Fig. 4C, ChIP analysis detected phosphorylated RelA/p65(S536) and SMRT (S2410) at 50 to 70 min, which preceded the detection of acetylated RelA/p65(K310) at 60 to 70 min by re-ChIP analysis. These results suggest that chromatin-associated RelA/p65 is phosphorylated at S536 before the protein becomes acetylated at K310. Once again, the detection of phos-

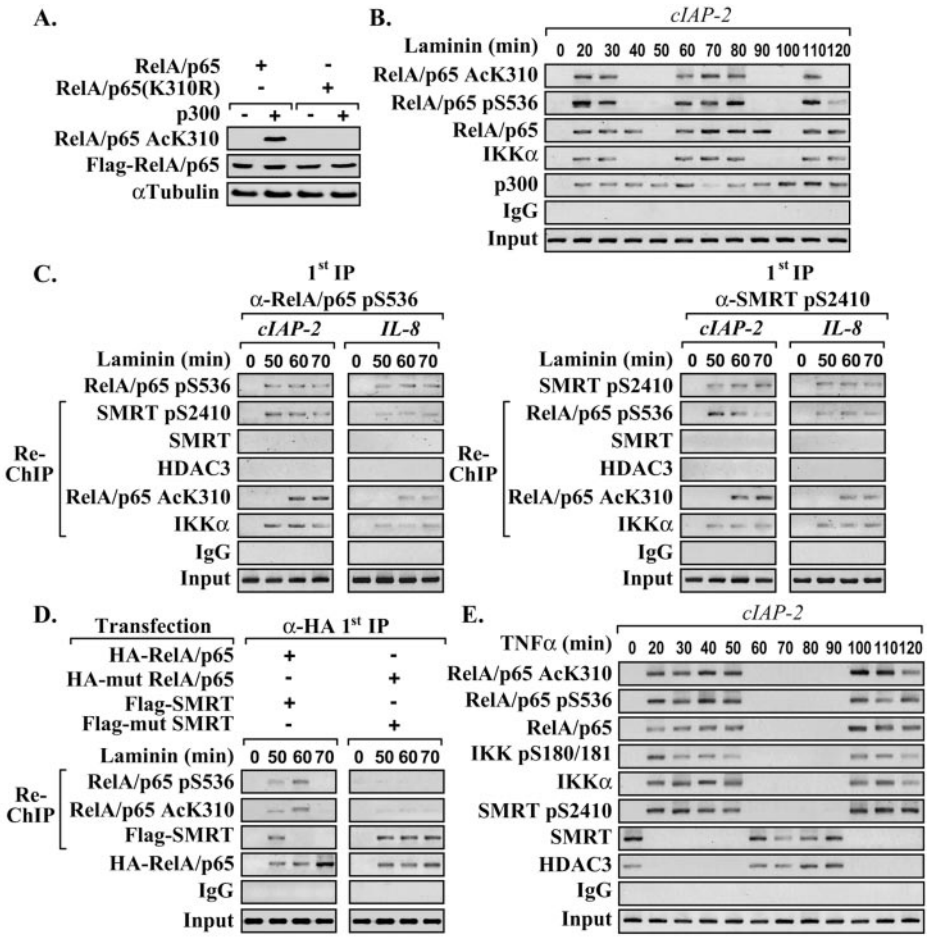

FIG. 4. IKKα-induced phosphorylation of RelA/p65(S536) precedes acetylation at K310. (A) Cell acetylation assays were performed to demonstrate the specificity of the anti-RelA/p65 K310 antibody. HEK 293T cells were transfected with plasmids encoding Flag-RelA/p65 or mutant Flag-RelA/p65(K310R) and with plasmids encoding either the p300 acetyltransferase or empty vector control. Western blot analysis was performed on total cell lysates, and acetylated RelA/p65 protein was detected using the newly developed anti-RelA/p65 AcK310 antibody. Western blots were stripped and reanalyzed using anti-Flag antibody, demonstrating equal amounts of Flag-RelA/p65 protein expression. (B) Chromatin-associated phosphorylation of RelA/p65(S536) corresponds with acetylation of RelA/p65(K310). ChIP analysis of the *cIAP-2* promoter was performed on DU145 cells following attachment to laminin. (C) Phosphorylation of RelA/p65 and SMRT corresponded to a loss of HDAC3 activity and an increase in RelA/p65 acetylation. Endogenous re-ChIP analysis was performed on both the *cIAP-2* and *IL-8* promoters following reattachment of DU145 cells to laminin. The immunoprecipitations for both ChIP and re-ChIP analysis were performed using either anti-RelA/p65 (α-RelA/p65) pS536 or anti-SMRT (α-SMRT) p2410 antibody. (D) The inability to phosphorylate SMRT and RelA/p65 governs the acetylation status of RelA/p65. HEK 293T cells were transfected with reduced amounts of plasmids (200 ng each/100-mm dish) encoding either wild-type HA-RelA/p65 and Flag-SMRT or mutant HA-RelA/p65(S536A) and Flag-SMRT(S2028,2410A); ChIP and re-ChIP analyses were performed following attachment to laminin as described for Fig. 3C. (E) TNF-α induces chromatin-associated RelA/p65(S536) and SMRT(S2410) phosphorylation which corresponds with RelA/p65(K310) acetylation on the *cIAP-2* promoter. Endogenous ChIP analysis on the *cIAP-2* promoter was performed using overnight serum-deprived DU145 cells stimulated with TNF-α (10 ng/ml).

phorylated RelA/p65(S536) corresponded with the recruitment of IKKα to chromatin. Although phosphorylated SMRT(S2410) remained physically associated with phosphorylated RelA/p65(S536) at 50 to 70 min, derepression of SMRT due to a loss of chromatin-bound HDAC3 was evident. Similar results were observed whether anti-RelA/p65(pS536) or anti-SMRT(p2410) antibody was used during the first immunoprecipitation of the re-ChIP analysis (Fig. 4C, right panel). These results are consistent with the hypothesis that RelA/p65 tethers the SMRT corepressor in a phosphorylated and inactive state during times of maximal NF-κB-mediated transcription. Collectively, these experiments provide the first evidence that IKKα contributes to RelA/p65(K310) acetylation, in part, by actively derepressing the SMRT-HDAC3 corepressor complex.

The re-ChIP analysis indicates that IKKα activity corresponds with phosphorylation of both chromatin-associated RelA/p65(S536) and SMRT(S2410). To determine whether it is the phosphorylation of RelA/p65 and SMRT and the loss of HDAC3 activity that were governing Rel/p65(K310) acetylation, we repeated re-ChIP analysis following ectopic expression of either the wild-type RelA/p65 and SMRT or mutant RelA/p65 and SMRT proteins as described previously for Fig. 3C. Cells expressing both wild-type RelA/p65 and SMRT proteins displayed chromatin-associated phosphorylated RelA/p65 (S536) and acetylated RelA/p65(K310) (Fig. 4D). However,

cells expressing both mutant RelA/p65(S536A) and SMRT (S2028,2410A), failed to display acetylated RelA/p65(K310) across the *cIAP-2* promoter, presumably because IKKα was no longer capable of derepressing SMRT-associated deacetylase activity. These results suggest that IKKα-induced phosphorylation of RelA/p65 and SMRT regulates NF-κB transcription by the ability of IKKα to actively derepress the SMRT-HDAC3 complex. Thus, our data support the conclusion that the combination of phosphorylation events that govern SMRT derepression from active NF-κB heterodimer allows p300 to acetylate RelA/p65(K310).

To determine whether this mode of regulation was stimulus dependent or a general mechanism that governs NF-κB-dependent transcription in response to other physiological stimuli, we repeated ChIP experiments using the TNF-α cytokine. As shown in Fig. 4E, the addition of TNF-α to DU145 cells stimulated chromatin-associated recruitment of active IKKα, which corresponded with phosphorylated RelA/p65(S536) and SMRT(S2410) proteins. As shown previously, following laminin attachment the addition of TNF-α stimulates the detection of the phosphorylated SMRT(S2410) protein, which inversely correlates with the detection of the unmodified SMRT and HDAC3 proteins on the *cIAP-2* promoter. Importantly, TNF-α-induced IKKα activity was associated with the accumulation of the chromatin-bound phosphorylated RelA/p65(S536) protein, a loss of SMRT-HDAC3 deacetylase activity, and the detection of the acetylated RelA/p65(K310) protein (Fig. 4E). Occupancy of phosphorylated RelA/p65(S536) and SMRT (S2410) species is associated with the loss of HDAC3 recruitment and subsequent acetylation of RelA/p65(K310). These results indicate that physiological stimuli, such as laminin attachment and TNF-α, stimulate NF-κB transcription through similar chromatin-mediated mechanisms.

Disruption of IKK activity prevents RelA/p65 phosphorylation and acetylation, blocking NF-κB transcription and cell survival. Data thus far suggest that there are distinct phases of SMRT derepression. In the first phase, IKKα is required to derepress SMRT from the basal repression complex, consisting of p50 homodimers, and in the latter phase, IKKα derepresses SMRT from the transcriptionally active p50-RelA/p65 heterodimer. Since siRNA knockdown of IKKα prevents the first phase of SMRT derepression (26), this approach could not be used to study the second phase of derepression on the p50-RelA/p65 heterodimer. Therefore, we decided to identify pharmacological inhibitors of IKK that could be added after the first phase of SMRT derepression had occurred. In this way, we could evaluate the requirement of IKKα for the latter phase of SMRT derepression on the transcriptionally active p50-RelA/p65 heterodimer (Fig. 5B). During our analysis, we screened five previously published IKK inhibitors (1, 27, 36, 44, 49, 53, 70). Many of these agents diminished IKK-induced phosphorylation of IκBα when added to cells prior to attachment on laminin. As shown in Fig. 5A, pretreatment of DU145 cells with Bay 11-7082 prior to laminin attachment inhibited IKK activity, as measured by immunokinase assays. However, Bay 11-7082 was the only compound that reproducibly inhibited SMRT(S2410) and RelA/p65(S536) phosphorylation after cells were replated on laminin. For this reason, we chose to use Bay 11 to determine the importance of IKK activity for SMRT derepression in the latter phase of NF-κB transcriptional activation.

To experimentally address this issue, DU145 cells were first replated on laminin (T0), and 30 min later, cells were treated with Bay 11 or empty vehicle control DMSO (Fig. 5B). Cells were harvested 30 min later (T60), and phospho-specific protein levels were detected by Western blot analysis (Fig. 5B). Alternatively, cells were harvested over the time course indicated, and ChIP analysis was performed to measure the requirement of IKK for chromatin-mediated effects (Fig. 5C). As shown in Fig. 5B, the addition of Bay 11 significantly diminished the ability of cells to express phosphorylated RelA/p65(S536) and SMRT(2410) following reattachment to laminin. The loss of phosphorylated RelA/p65(S536) did not inhibit nuclear translocation of RelA/p65, indicating that the addition of Bay 11 at 30 min postattachment was targeting IKK-mediated phosphorylation of RelA/p65 rather than preventing the phosphorylation and degradation of the IκBα inhibitor protein. This was reinforced when the addition of Bay 11 30 min following attachment to laminin did not prevent IKK-mediated IKKα degradation (Fig. 5B). These results support the use of Bay 11 as a selective IKK inhibitor that is immediately effective when added 30 min following attachment of cells to laminin. In this way, we can study the requirement of IKK activity for the latter phase of NF-κB transcription to determine the importance of IKK for RelA/p65 and SMRT phosphorylation and RelA/p65 acetylation.

To elucidate the importance of IKK activity for the second phase of derepression, Bay 11-treated cells were analyzed by ChIP analysis over the time course indicated. Similar to untreated cells (Fig. 1 and 4), DMSO-treated control cells displayed phosphorylated and acetylated RelA/p65 on the *cIAP-2* promoter following attachment to laminin (Fig. 5D). IKKα was recruited to chromatin over this same time frame, and detection of the IKKα protein with the anti-IKK pS180/181 antibody indicated that the kinase was active. Consistent with IKKα-dependent regulation, we detected an inverse correlation between chromatin-associated phosphorylated SMRT(S2410) and total SMRT and HDAC3 protein on the *cIAP-2* promoter. The addition of Bay 11 to cells 30 min following attachment did not disrupt the initial IKKα activity required for SMRT derepression from p50 homodimers, and yet it effectively inhibited IKKα activity in the second phase of derepression, as indicated by the delay in recruitment of IKKα and IKK(S180/181) to chromatin (Fig. 5D). In contrast to control cells, Bay 11 treatment delayed IKKα recruitment and kinase activity, shifting the kinetics from 50 to 70 min to 70 to 90 min (Fig. 5B). The ability of Bay 11 to delay IKKα activity on chromatin had significant repercussions on NF-κB activity by delaying and disrupting phosphorylated RelA/p65(S536) and phosphorylated SMRT(S2410). Moreover, in Bay 11-treated cells, the SMRT and HDAC3 proteins remained associated with the *cIAP-2* promoter despite replating cells on laminin. The addition of Bay 11 following laminin attachment did not block RelA/p65 nuclear translocation or chromatin association but rather potentiated further the interaction of the transcription factor with chromatin. Consistent with the hypothesis that IKKα is required for SMRT-HDAC3 derepression, Bay 11-treated cells no longer displayed RelA/p65(K310) acetylation (Fig. 5D). The presence of unmodified RelA/p65, SMRT, and

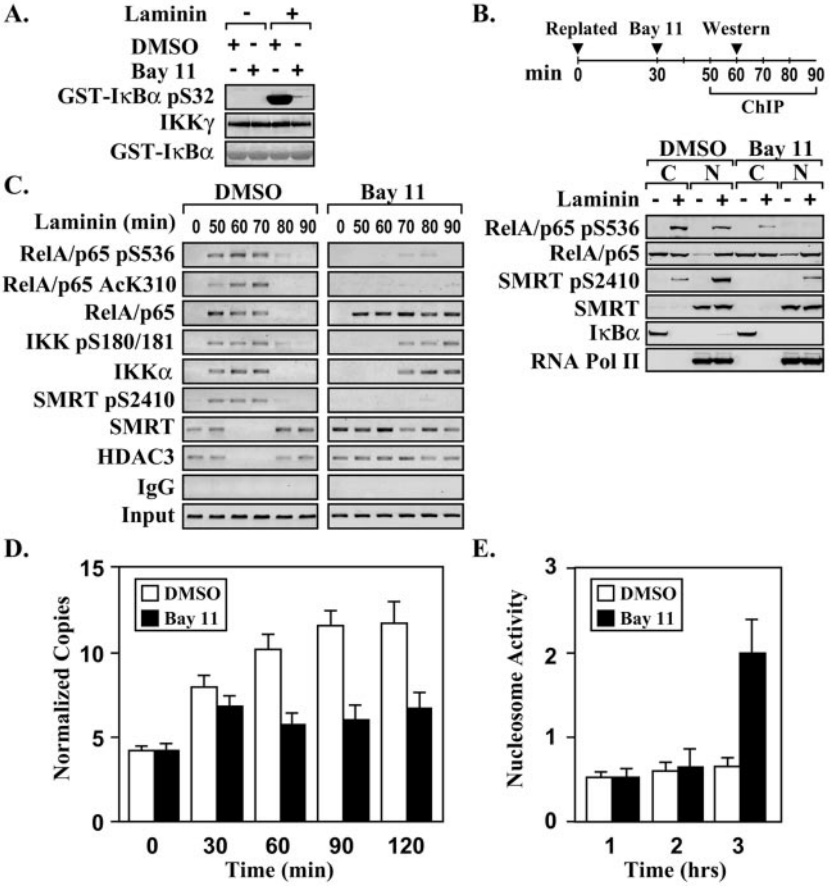

FIG. 5. IKK activity is required for SMRT derepression, full NF-κB transcription, and cell survival. (A) Bay 11 is an effective pharmacological inhibitor of IKK activity in response to laminin attachment. DU145 cell suspensions were incubated with either DMSO or Bay 11 for 5 min and then plated on laminin-coated plates. Thirty minutes following replating, cells were harvested and immunokinase reactions were carried out to measure IKK activity. IKK activity was immunoprecipitated using anti-IKKγ antibody, and immunokinase reactions were performed by mixing immunoprecipitates with GST-IκBα(1–54) protein and ATP. Western blot analysis was performed, using anti-IκBα(S32) phospho-specific antibody as a measurement of IKK activity. Blots were reanalyzed using anti-IKKγ antibody to demonstrate efficient immunoprecipitation. Polyacrylamide gel was Coomassie stained prior to transfer to show relative levels of GST-IκBα proteins. (B) Top panel: an illustration describing the design of Bay 11 treatment postattachment. DU145 cells were replated on laminin (T0). Thirty minutes later, cells were treated either with the DMSO vehicle control or with Bay 11. Cells were harvested at T60, and cytosolic and nuclear extracts were harvested and analyzed by Western blot analysis as shown in panel B. Alternatively, cells were harvested at T50 to T90 and ChIP analysis was performed as shown in C. Bottom panel: treatment of DU145 cells with Bay 11 results in diminished levels of phosphorylated RelA/p65(S536) and SMRT(S2410) protein in cytoplasmic (C) and nuclear (N) extracts. Detection of RNA polymerase II protein served as a nuclear loading control for Western blot analysis. (C) Inhibition of IKK activity blocked phosphorylation and acetylation of RelA/p65 and converted RelA/p65 to an active repressor by tethering the SMRT-HDAC3 complex. DU145 cells were treated with either DMSO or Bay 11 as described for panel B. ChIP analysis was performed using PCR primers corresponding to the *cIAP-2* promoter. Ten percent of each sonicated sample was included as a loading input control. (D) Disruption of chromatin-associated IKKα using Bay 11 inhibits NF-κB transcription of the *cIAP-2* gene. DU145 cells were replated and treated with either Bay 11 or DMSO as described for panel B. Total RNAs were isolated, and *cIAP-2* transcripts were quantitated using PCR. Transcripts encoding the housekeeping gene *GUS* served as an internal RNA control during the real-time PCR. Total mRNA transcripts were calculated according to previous methods described in Materials and Methods. Data represent the mean ± standard deviation for two independent experiments performed in duplicate. (E) Prolonged exposure of postreattached cells to the IKK inhibitor Bay 11 predisposed cells to apoptotic cell death. DU145 cells were replated and treated with Bay 11 or DMSO, and cells were harvested (1, 2, and 3 h) following reattachment. Nucleosome formation was measured as an indicator of apoptosis using the Cell Death Detection enzyme-linked immunosorbent assay (Roche). All absorbance values were normalized to the amount of total protein. Experiments shown represent the means ± standard deviations for three independent experiments performed in triplicate.

HDAC3 in the Bay 11-treated cells suggests that disruption of IKK activity results in active repression. Active repression through NF-κB has recently been described as a process whereby DNA-damaging pathways involving ARF-induced activation of ATR and Chk1 block NF-κB-mediated transcription by converting RelA/p65 from an activator to a repressor (13, 51, 52).

To address whether Bay 11 was indeed inhibiting *cIAP-2* transcription, cells were either harvested (T0) or replated on laminin and 30 min later treated with either Bay 11 or DMSO. Total RNAs were isolated following replating on laminin (T30 to T120), and *cIAP-2* transcripts were quantitated using real-time PCR. As shown in Fig. 5C, the addition of Bay 11 inhibited *cIAP-2* gene expression compared to results with vehicle

control-treated cells. Similar results were obtained following real-time PCR analysis of the *IL-8* transcripts (data not shown). Since NF-κB transcriptional activity is required for cell survival following attachment to laminin (26), we analyzed whether inhibition of chromatin-associated IKK activity by Bay 11 could sensitize cells to apoptosis. Cells were treated with either DMSO or Bay 11 as described in for Fig. 5C, cells were harvested, and nucleosome activities were measure. As predicted, the inhibition of the second phase of IKKα activity by Bay 11 disrupted NF-κB transcription, sensitizing the cells to apoptosis despite binding to laminin (Fig. 5D). Collectively, experiments described here show that the pharmacological inhibitor Bay 11 delayed chromatin-associated IKKα activity, causing a discordant regulation such that neither RelA/p65(S536) nor SMRT(S2410) became phosphorylated by IKKα. The inability of RelA/p65(S536) and SMRT(S2410) to become phosphorylated inhibited derepression from the SMRT-HDAC3 complex, thus preventing RelA/p65 acetylation and NF-κB transcription. These results demonstrate the importance of IKKα-mediated phosphorylation of RelA/p65 and SMRT and suggest that the inhibition of IKKα may cause active repression and premature termination of NF-κB transcription.

DISCUSSION

Many different physiological stimuli, including engagement of cytokine and T-cell receptors, DNA damage stress responses, and extracellular matrix/integrin attachment, activate a plethora of signaling cascades that mediate NF-κB transcription (22, 25, 26). Despite the numerous avenues for NF-κB activation, many of these pathways converge on the IKK signalosome complex, indicating the importance of IKK activation for NF-κB transcription and biological responsiveness. NF-κB becomes activated through at least two different IKK signalosome complexes. In the classical NF-κB pathway, the IKK complex is composed of IKKα, IKKβ, and IKKγ, while the alternative NF-κB pathway involves the NIK-dependent activation of the IKKα homodimer (25). In the classical pathway, IKKβ is critical for phosphorylation of IκBα and IκBβ, which governs nuclear translocation of the RelA/p65-p50 heterodimer. Unlike IKKβ, IKKα has distinct functions as a nuclear kinase capable of phosphorylating SRC-3 (66), CBP (67), histone H3, and the SMRT corepressor (4, 5, 26, 67), events linked to transcriptional activation of both NF-κB-regulated and non-NF-κB-regulated gene targets (4, 5, 26, 47).

Our laboratory recently demonstrated that IKKα is critical for derepressing NF-κB-regulated promoters by phosphorylating and inactivating the SMRT-HDAC3 repressor complex from the chromatin-bound p50 homodimer (26). This event is critical for NF-κB transcription, because derepression of SMRT and HDAC3 relieves the basal repression complex, allowing recruitment of the transcriptionally competent p50-RelA/p65 heterodimer (Fig. 6). Since IKKα has been shown to phosphorylate RelA/p65(S536) (30, 34, 46), in the current study we examined whether IKKα potentiates NF-κB transcription at the chromatin level by phosphorylating both RelA/p65 and SMRT. This is an important question that examines whether IKKα controls NF-κB transcription by regulating derepression of the SMRT-HDAC3 complex from both basal and active components of NF-κB. Using ChIP and re-ChIP analysis, we found that RelA/p65 phosphorylation at S536 corresponds with IKKα activity on the *cIAP-2* promoter upon attachment to laminin or following the addition of TNF-α. In an extended time course, our studies demonstrate that chromatin-bound IKKα but not IKKβ corresponds with RelA/p65(S536) phosphorylation during times of maximal NF-κB transcription. However, based on previous studies that support a role of IKKβ-induced phosphorylation of RelA/p65 (68), we cannot exclude the possibility that IKKβ-mediated phosphorylation of RelA/p65(S536) occurs in the cytoplasm and is important during initial recruitment of RelA/p65 to chromatin. Importantly, we also found that IKKα mediates a similar phosphorylation event on the SMRT corepressor at S2410 when bound to the transcriptionally active RelA/p65 component of NF-κB. Re-ChIP analysis confirms that chromatin-bound IKKα coordinates the simultaneous phosphorylation of RelA/p65(S536) and SMRT(S2410). Although phosphorylated SMRT remains bound to RelA/p65, derepression of SMRT is evidenced by the loss of chromatin-associated HDAC3 activity. The resulting consequences are that phosphorylation of RelA/p65(S536) and SMRT(S2410) and the loss of HDAC3 activity occur prior to acetylation of RelA/p65 at K310. These results support the hypothesis that IKKα-induced phosphorylation of RelA/p65(S536) displaces corepressor activity, allowing p300 to induce acetylation of RelA/p65 (Fig. 6). Simultaneous phosphorylation of RelA/p65 and SMRT is critical for NF-κB transcription, since disruption of IKKα activity blocked phosphorylation of these molecules, disrupted HDAC3 derepression, and prevented p300-mediated acetylation of RelA/p65. Our work indicates that IKKα-induced phosphorylation within the transactivation domain of RelA/p65(S536) deactivates SMRT-HDAC3 repressor complexes to allow RelA/p65 to become acetylated by p300.

Consistent with our results, Chen and colleagues recently demonstrate that IKK-mediated RelA/p65(S536) phosphorylation regulates K310 acetylation (17). The authors provide evidence that the phosphorylation status of RelA/p65 at either S276 or S536 is required for recruitment of p300 and subsequent acetylation of RelA/p65(K310). Although we have not evaluated the phosphorylation status of RelA/p65(S276) in our model system, the use of nonphosphorylatable mutants of the RelA/p65(S336A) and SMRT(S2028,2410A) proteins or the inhibition of IKK activity results in active repression of NF-κB promoters by tethering the SMRT-HDAC3 complex. The interpretation of our results is similar to work published by Zhong and colleagues (72), which demonstrated that phosphorylation of RelA/p65(S276) governs an exchange in which HDAC1 becomes derepressed and CBP/p300 is recruited to active RelA/p65. Therefore, in agreement with previously published work (17, 20, 72), it is likely that phosphorylation of RelA/p65 at S276, S536, and possibly S311 and S529 activates RelA/p65 by first derepressing HDAC1 and SMRT-HDAC3 repression complexes, allowing subsequent recruitment of CBP/p300 and acetylation of RelA/p65(K310). This argument suggests that there are multiple phosphorylation sites within RelA/p65 that seem to function in a similar manner to mediate derepression of HDAC-containing complexes and recruitment of CBP/p300. This argument is supported by the fact that NF-κB is a ubiquitous transcription factor that must respond to

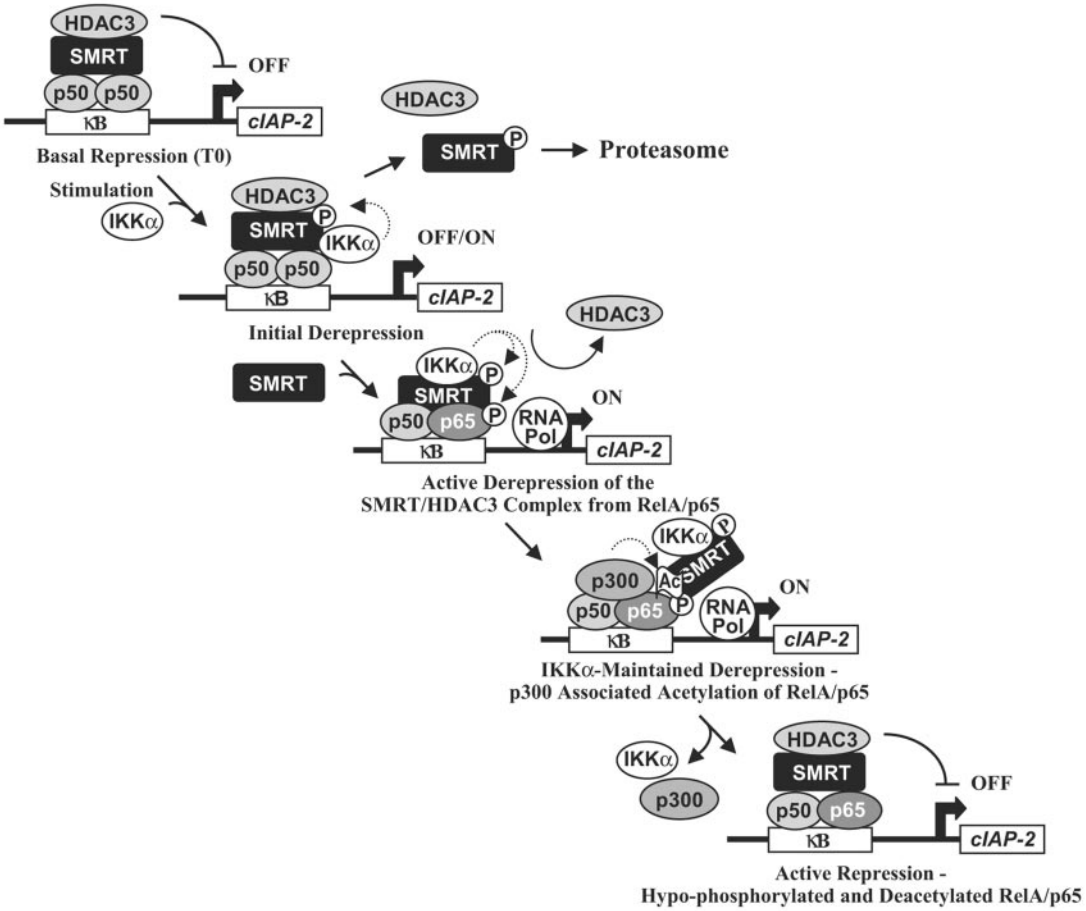

FIG. 6. IKKα-mediated derepression of NF-κB-regulated genes occurs in distinct phases. Following cellular stimulation, IKKα is responsible for the removal of the SMRT-HDAC3 complex tethered by p50 homodimers. The SMRT-HDAC3 complex is responsible for basal repression of classical NF-κB-regulated gene targets in the unstimulated state (26). Upon stimulation, IKKα-mediated phosphorylation of SMRT initiates derepression to allow the active p50-RelA/p65 heterodimer of NF-κB to bind and potentiate transcription. However, almost immediately after the active p50-RelA/p65 heterodimer binds to the promoter, the SMRT corepressor returns to the chromatin-bound NF-κB complex. At this point there are at least two possible outcomes. If IKKα activity is inhibited, SMRT tethers HDAC3 for immediate active repression of NF-κB transcription. However, in the context of the appropriate stimuli, IKKα remains chromatin associated and phosphorylates both RelA/p65(S536) and SMRT(S2410). It is this dual phosphorylation event which prevents HDAC3 recruitment and chromatin association to the NF-κB-regulated promoter. Displacement of HDAC3 from active NF-κB allows p300 to load and subsequently acetylate RelA/p65 at K310, which is required for full NF-κB transcription. Over a 2-h period, IKKα-mediated derepression of the SMRT-HDAC3 complex is repeated, and each time, p300-mediated acetylation is observed. Thus, IKKα governs a check-and-balance system in which phosphorylation of SMRT(S2410) and RelA/p65(S536) mediates the loss of HDAC3 activity. It is this derepression event that allows RelA/p65 to become acetylated to insure appropriate NF-κB-mediated transcription.

many different physiological stimuli. Unlike NF-κB, many corepressors and HDAC enzymes display tissue-specific differences in protein expression (19, 62). Therefore, NF-κB has evolved such that different NF-κB stimuli activate selective combinations of serine/threonine kinases capable of phosphorylating RelA/p65 within the RHD at S276 or within the transactivation domain at S536 to derepress HDAC1 or SMRT-HDAC3 complexes. Derepression of HDAC1 or SMRT-HDAC3 from RelA/p65 has the same physiological response in the end: CBP/p300 recruitment and RelA/p65(K310) acetylation. However, even the specificities of these two HDAC complexes can be quite different. For example, our laboratory and others have found that HDAC1 directly interacts with RelA/p65 and that this occurs even in the absence of the SMRT or N-CoR corepressor (6, 72). Since HDAC1 is associated with many different corepressor complexes, including the Sin3-SAP, NURD, NURD-related, and CoREST-HDAC complexes (29), it is possible that HDAC1 has the potential to directly recruit these corepressor complexes to NF-κB. In contrast, HDAC3 does not directly interact with RelA/p65 but rather is tethered by either SMRT or N-CoR (7, 26, 35). However, unlike HDAC1, HDAC3 has the potential to recruit members of the class II HDACs to the repression complex (21). Finally, not only are there multiple corepressor complexes within the same cell that bind and regulate NF-κB activity, but there seem to be promoter-specific preferences for certain repression complexes (7, 26). Therefore, derepression of various corepressor complexes associated with NF-κB probably depends on multiple parameters, including stimulus-dependent activation of serine/threonine kinases, promoter occupancy, and tissue specificity.

Although the current study and recently published work (17) help to address the significance of RelA/p65 S536 phosphorylation, there are still many unanswered questions about SMRT derepression from active NF-κB transcriptional complex. Re-ChIP analysis indicates that phosphorylated SMRT(S2410) remains tethered to chromatin-bound phosphorylated RelA/p65(S536). Using in vitro kinase interaction assays, we demonstrated that phosphorylation of RelA/p65(S536) or RD I/II of SMRT(S2410) causes a disassociation between these two portions of the molecules. Since it is difficult to express full-length GST-SMRT proteins, we were unable to test whether a similar mechanism occurs with both of the full-length SMRT and RelA/p65 proteins. However, we have shown that RelA/p65 also interacts with SMRT in another region of the protein (amino acids 1031 to 1596) (Fig. 2A). IKKα is capable of phosphorylating SMRT within the same C-terminal domain that encompasses the RD I/II domain (26). However, in in vitro kinase interaction assays, we were unable to displace the interaction of these two proteins (J. E. Hoberg and M. W. Mayo, data not shown). Based on this observation, we believe that endogenous phosphorylated SMRT may still interact with phosphorylated RelA/p65 through this domain.

Consistent with IKKα-induced phosphorylation and derepression of SMRT, HDAC3 is no longer associated with phosphorylated or acetylated RelA/p65. However, the preservation of the phosphorylated SMRT species on RelA/p65 at NF-κB-regulated promoters suggests either that this is a highly dynamic process where SMRT cycles between phosphorylation and derepression from NF-κB or that SMRT plays an ancillary role in the transcription process where it is bound in a derepressed state poised for reactivation. During our initial characterization of IKKα-mediated derepression of the SMRT corepressor complex (26), we found that SMRT returns to chromatin prior to HDAC3 binding. Moreover, chromatin-associated SMRT is associated with the recruitment of the TCP-1 protein, a member of the TCP-1 ring complex (TRiC) responsible for loading HDAC3 onto SMRT (24). These results suggest that the TRiC may assemble HDAC3 onto chromatin-bound SMRT. Therefore, it is possible either that the phosphorylated SMRT that we detect bound to RelA/p65 is not yet targeted for HDAC3 reloading by the TRiC or that IKKα-induced phosphorylation prevents the reestablishment of the repressor complex on NF-κB promoters.

In addition to HDAC3 loading issues, it is important to understand better how IKKα phosphorylation alters SMRT such that it no longer is capable of binding HDAC3. It is possible that IKKα-induced phosphorylation of RelA/p65 and potentially histone H3 alters the binding and/or conformation of the SMRT corepressor, thus potentiating derepression. SMRT contains two HDAC3 interaction domains. Perhaps the more important of these two regions resides in the SANT (SWI3/ADA2/N-CoR/TFIIB)-like domain of SMRT. SMRT contains a pair of SANT motifs; the N-terminal-most domain, called the deacetylase activation domain (DAD), which is required to bind and activate HDAC3 (23), and the histone interaction domain (HID). The significance of the HID is that it has been shown to increase the enzymatic activity of HDCA3 by lowering the K_m for histone substrates (71). Therefore, the DAD and HID motifs work together synergistically to promote acetylation of core histones. Interestingly, the HID binds preferentially to unacetylated histone substrates and is inhibited by acetylated histone substrates. Based on this understanding, the HID has been proposed to function in a "feed-forward" mechanism that may interpret the histone code (71). The presence of the hydrophobic groove in the DAD is required to bind and activate HDAC3 in conjunction with the HID (18); it is conceivable that posttranslational modifications to SMRT alter the structure of the SANT domains such that they can no longer recruit HDAC3 or bind core histones. This change in the structure of SMRT may also be regulated by the acetylation status of both RelA/p65 and histone tails associated with nucleosome surrounding the NF-κB-regulated promoter. This hypothesis is supported by several observations. First, the HID domain does not preferentially recognize acetylated core histones (71), which would be predicted to affect greatly the ability of DAD to bind and activate HDAC3. Second, as expected, during full NF-κB-mediated transcription, histone H3 and H4 are hyperacetylated (4, 26, 67, 69), as is RelA/p65 (17 and Fig. 4). Finally, in this study we provide evidence that IKKα-induced phosphorylation of SMRT within RD I/II displaces interaction with RelA/p65 (Fig. 2). Together these observations provide support for the idea that phosphorylated SMRT and RelA/p65, as well as the acetylation of core histones, may change the secondary or tertiary structure of SMRT such that the DAD no longer binds and activates HDAC3. Work currently ongoing in the laboratory will address this hypothesis.

Finally, another interesting observation from our studies is that the RelA/p65 and IKKα proteins are not static but rather cycle on and off of chromatin during NF-κB transcription. It is well established that both NF-κB and SMRT cycle between the cytoplasm and the nucleus in response to physiological stimuli (15, 29). One potential explanation is that NF-κB becomes downregulated by the resynthesis of the IκBα proteins, which bind back to chromatin-associated RelA/p65 and facilitate the nuclear export of NF-κB (15, 16). Although it is clear that resynthesized IκBα does help redistribute NF-κB into the cytoplasm (16, 50), in this study chromatin-bound IκBα does not correspond with the cyclic phases of RelA/p65 binding to the *cIAP-2* promoter. Perhaps a better explanation is that promoter-bound RelA/p65 is posttranslationally modified directly on chromatin and stimulated for degradation through a proteasome-dependent mechanism. RelA/p65 is known to be degraded through ubiquitin-dependent mechanisms (54), and NF-κB transcription has been proposed to be terminated through ubiquitin-mediated targeting of chromatin-bound RelA/p65 (55). The turnover of transcriptionally active NF-κB through a ubiquitin-dependent process is an attractive mechanism, since we have shown that SMRT-mediated turnover is TBL1/TBLR1/Ubc5 dependent (26). Thus, it will be important to identify the posttranslational mechanisms governing chromatin-bound RelA/p65 and to elucidate how ubiquitin and/or ubiquitin-like processes regulate the turnover of RelA/p65 and IKKα to terminate NF-κB transcription.

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