THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 29, pp. 21308–21318, July 20, 2007

Promoter-dependent Effect of IKKα on NF-κB/p65 DNA Binding*

Received for publication, November 20, 2006, and in revised form, May 2, 2007 Published, JBC Papers in Press, May 30, 2007, DOI 10.1074/jbc.M610728200

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IKKα regulates many chromatin events in the nuclear phase of the NF-κB program, including phosphorylation of histone H3 and removal of co-repressors from NF-κB-dependent promoters. However, all of the nuclear functions of IKKα are not understood. In this study, using mouse embryonic fibroblasts IKKα knock-out and reexpressing IKKα after retroviral transduction, we demonstrate that IKKα contributes to NF-κB/p65 DNA binding activity on an exogenous κB element and on some, but not all, endogenous NF-κB-target promoters. Indeed, p65 chromatin immunoprecipitation assays revealed that IKKα is crucial for p65 binding on κB sites of *icam-1* and *mcp-1* promoters but not on *iκbα* promoter. The mutation of IKKα putative nuclear localization sequence, which prevents its nuclear translocation, or of crucial serines in the IKKα activation loop completely inhibits p65 binding on *icam-1* and *mcp-1* promoters and rather enhances p65 binding on the *iκbα* promoter. Further molecular studies demonstrated that the removal of chromatin-bound HDAC3, a histone deacetylase inhibiting p65 DNA binding, is differentially regulated by IKKα in a promoter-specific manner. Indeed, whereas the absence of IKKα induces HDAC3 recruitment and repression on the *icam-1* promoter, it has an opposite effect on the *iκbα* promoter, where a better p65 binding occurs. We conclude that nuclear IKKα is required for p65 DNA binding in a gene-specific manner.

Nuclear factor-κB (NF-κB)[5] is a key transcription factor involved in the expression of genes regulating innate and adaptive immunity (1), cellular proliferation, survival (2, 3), and development (4). NF-κB consists of homo- or heterodimers of a group of five proteins, namely NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), p65/RelA, c-Rel, and RelB (5). In unstimulated cells, NF-κB is sequestered in the cytoplasm through its tight association with inhibitory proteins of the IκB family, comprising notably IκBα (5). Upon cellular stimulation with proinflammatory cytokines, lipopolysaccharide, antigens or viral products, the so-called classical NF-κB activation pathway is engaged. IκBα is rapidly phosphorylated on Ser^{32} and Ser^{36}, which triggers its polyubiquitination and subsequent degradation by the 26 S proteasome. The freed NF-κB then translocates into the nucleus, where it binds κB sites and enhances transcription of a large number of genes implicated notably in the inflammatory response (5). Phosphorylation of IκBα on Ser^{32} and Ser^{36} is achieved by the IκB kinase (IKK) complex, which includes the scaffold protein NF-κB essential modulator (NEMO; also called IKKγ) (6) and the IKKα and IKKβ kinases (7). A novel NEMO-independent NF-κB-activating pathway was recently described. This alternative pathway is engaged notably upon lymphotoxin-β or B cell-activating factor induction and enhances NF-κB-inducing kinase- and IKKα-dependent processing of p100 into p52 (8, 9). This subunit binds DNA in association with its partners and stimulates transcription of genes important for secondary lymphoid organ development, B cell homeostasis, and adaptive immunity (1, 10). IKK-independent phosphorylation of IκBα on Tyr^{42} has also been reported upon sodium pervanadate or hypoxia/reoxygenation treatment (11–13). This tyrosine phosphorylation is mediated by c-Src tyrosine kinase and triggers dissociation/degradation of IκBα from NF-κB complexes, allowing NF-κB to translocate into the nucleus (12, 14). Generation of knock-out mice for IKK complex subunits has revealed that IKKβ is the main kinase responsible for IκBα Ser^{32} and Ser^{36} phosphorylation and that NEMO/IKKγ assembles the IKKs into a functional kinase complex upon the classical pathway (15–18). On the other hand, whereas IKKα is crucial for p100 phosphorylation and subsequent NF-κB activation through the alternative pathway (10), in most cases this kinase is not required for the signal-induced phosphorylation of IκBα in the cytoplasm. Nevertheless, an optimal induction of NF-κB-dependent genes through the classical pathway appears to rely on the nuclear translocation of IKKα (19). Indeed, once

* This work was supported in part by the IAP 5/12 program, the "Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium) and the "Fonds Anti-cancéreux près l'Université de Liège. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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[5] The abbreviations used are: NF-κB, nuclear factor-κB; IKK, IκB kinase; HDAC, histone deacetylase; SMRT, silencing mediator for retinoic acid and thyroid hormone receptor; TNF-α, tumor necrosis factor-α; ChIP, chromatin immunoprecipitation assay; MEF, mouse embryonic fibroblast; WT, wild-type; NLS, nuclear localization sequence; EMSA, electrophoretic mobility shift assay; HIV-1, human immunodeficiency virus, type 1; Pv, sodium pervanadate; TSA, trichostatin A; KO, knock-out; CREB, cAMP-response element-binding protein.

in the nucleus, IKKα acts in a process called derepression that allows full NF-κB-mediated transcription by removing repressor complexes, such as SMRT and HDAC3, from target promoters (20). IKKα also phosphorylates histone H3, a component of nucleosomes. This phosphorylation triggers subsequent acetylation of histone H3 on Lys^{14} by the IKKα-associated histone acetyltransferase CREB-binding protein, a crucial step in modulating chromatin accessibility at NF-κB-responsive promoters (21–23). Besides a role of nuclear IKKα in positively regulating NF-κB-dependent gene transcription, IKKα also limits NF-κB activation in LPS-stimulated macrophages by mediating p65 and c-Rel turnover through phosphorylation of their C-terminal part (24). Some authors have also suggested that IKKα contributes to direct p65 DNA binding, since electrophoretic mobility shift assay (EMSA) experiments using *ikkα*$^{-/-}$ MEFs or HeLa cells transfected with IKKα small interfering RNA exhibit a clear inhibition of NF-κB binding activity upon TNF-α treatment, despite a quite unaltered IκBα phosphorylation/degradation (25, 26). However, these results seemed inconsistent with other reports (27, 28), including recent data obtained by p65 chromatin immunoprecipitation (ChIP) analysis on several NF-κB target genes in *ikkα*$^{-/-}$ MEFs (21, 23). Such a discrepancy reflects the need to determine whether or not a general mechanism can be drawn for all genes or whether some promoter-specific effects occur.

Here, we have addressed the role of IKKα in the control of NF-κB/p65 DNA binding in mouse embryonic fibroblasts lacking IKKα or complemented with either wild-type (WT) or mutant IKKα upon TNF-α stimulation. We showed that IKKα is required for p65 DNA binding on specific, but not all NF-κB-dependent, promoters. Indeed, p65 ChIP assays revealed that IKKα is crucial for p65 binding on κB sites of the *icam-1* (intercellular adhesion molecule-1) and *mcp-1* (monocyte chemoattractant protein-1) promoters but not on the *iκbα* promoter. This binding requires IKKα catalytic activity and an intact nuclear localization sequence (NLS), suggesting that IKKα acts in the nucleus to mediate its effects. Further molecular studies demonstrated that IKKα modifies HDAC3 recruitment in a promoter-specific manner, thereby explaining its gene-specific activity.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—MEFs were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% of heat-inactivated fetal calf serum and 2 mM L-glutamine. Antibodies against p65 and HDAC3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (sc-109 for p65 ChIP and Western blotting, sc-372G for p65 immunocytochemistry, and sc-11417 for HDAC3 ChIP). The antibodies against IKKα were from Pharmingen (for Western blotting) and Santa Cruz Biotechnology (sc-7182 for immunocytochemistry). Antibody against IKKβ was from Upstate Biotechnology (catalog number 05-035). Other antibodies were from StressGen (HSP60) and BD Transduction Laboratories (NBS1). Antibody against IκBα was a gift from R. Hay (St. Andrews, UK). Alexa Fluor 488 donkey anti-goat and 546 goat anti-rabbit antibodies were from Molecular Probes. Human recombinant TNF-α was from Peprotech, trichostatin A (TSA) was from Sigma, and SYBR green PCR master mix was from Applied Biosystems. Sodium pervanadate was prepared as described (29).

Plasmids, Site-directed Mutagenesis, and Retroviral Gene Transfer—pMX retroviral vector containing human wild-type *ikkα* and *ikkα AA* were previously described (8). The IKKα NLS mutant was generated by site-directed mutagenesis (Stratagene) by replacing Lys^{235}, Lys^{236}, and Lys^{237} of WT IKKα with alanines. These constructs were transfected by the calcium phosphate method into 293T cells in combination with the pEC ampho expression vector (8). Two days later, supernatants were collected and filtered prior to the transduction of *ikkα*$^{-/-}$ MEFs, and IKKα expression was confirmed by Western blotting.

Western Blotting and EMSA—Cytoplasmic and nuclear extracts were prepared as previously described (30). Cytoplasmic extracts were analyzed by Western blotting as described (31). Nuclear extracts and EMSA experiments were carried out as described (31) using ^{32}P-labeled oligonucleotide probes (Eurogentec) corresponding to the κB site of the HIV-1 long terminal repeat.

Immunocytochemistry and Confocal Microscopy—p65 and IKKα nuclear translocation was visualized by confocal microscopy. To visualize p65, TNF-α-treated MEFs were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. After washing with phosphate-buffered saline containing 10% fetal calf serum, slides were incubated with goat anti-p65 antibody (sc-372G; 1:100 dilution; Santa Cruz Biotechnology) for 1 h at 37 °C and then washed and incubated with Alexa Fluor 488 donkey anti-goat antibody (1:400 dilution) for 1 h at 37 °C. The slides were then washed, incubated with propidium iodide to visualize nuclei, mounted, and analyzed with a Leica TCS SP2 confocal microscope (Van Hopplynus). For IKKα immunolocalization, slides were incubated in 100% methanol at −20 °C for 6 min and then washed with phosphate-buffered saline and blocked in phosphate-buffered saline containing 0.5% gelatin (Bio-Rad) and 0.25% bovine serum albumin (Invitrogen). Then cells were incubated with the primary antibody (sc-7182; 1:100 dilution; Santa Cruz Biotechnology) in blocking solution overnight at 4 °C. After washing, cells were incubated with Alexa Fluor 546 goat anti-rabbit antibody (1:100 dilution) for 2 h at room temperature. Samples were mounted and analyzed with a LSM 510 Zeiss confocal microscope.

Chromatin Immunoprecipitation Assay—ChIP assays were carried out, and the solutions were prepared in our laboratory following the Upstate Cell Signaling protocol. After cross-linking with formaldehyde, treated cells were lysed and sonicated such that DNA fragments were 200–1,000 base pairs in length. After a preclear with protein A-agarose beads saturated with herring sperm DNA (Sigma), extracts were incubated overnight with a rabbit polyclonal anti-p65 antibody (sc-109; 2 μg; Santa Cruz Biotechnology) or HDAC3 antibody (sc-11417; 2 μg; Santa Cruz Biotechnology). To take into account aspecific binding to the beads, a treated extract was also incubated with an irrelevant antibody (anti-FLAG; M2; Sigma). The next day, precipitation

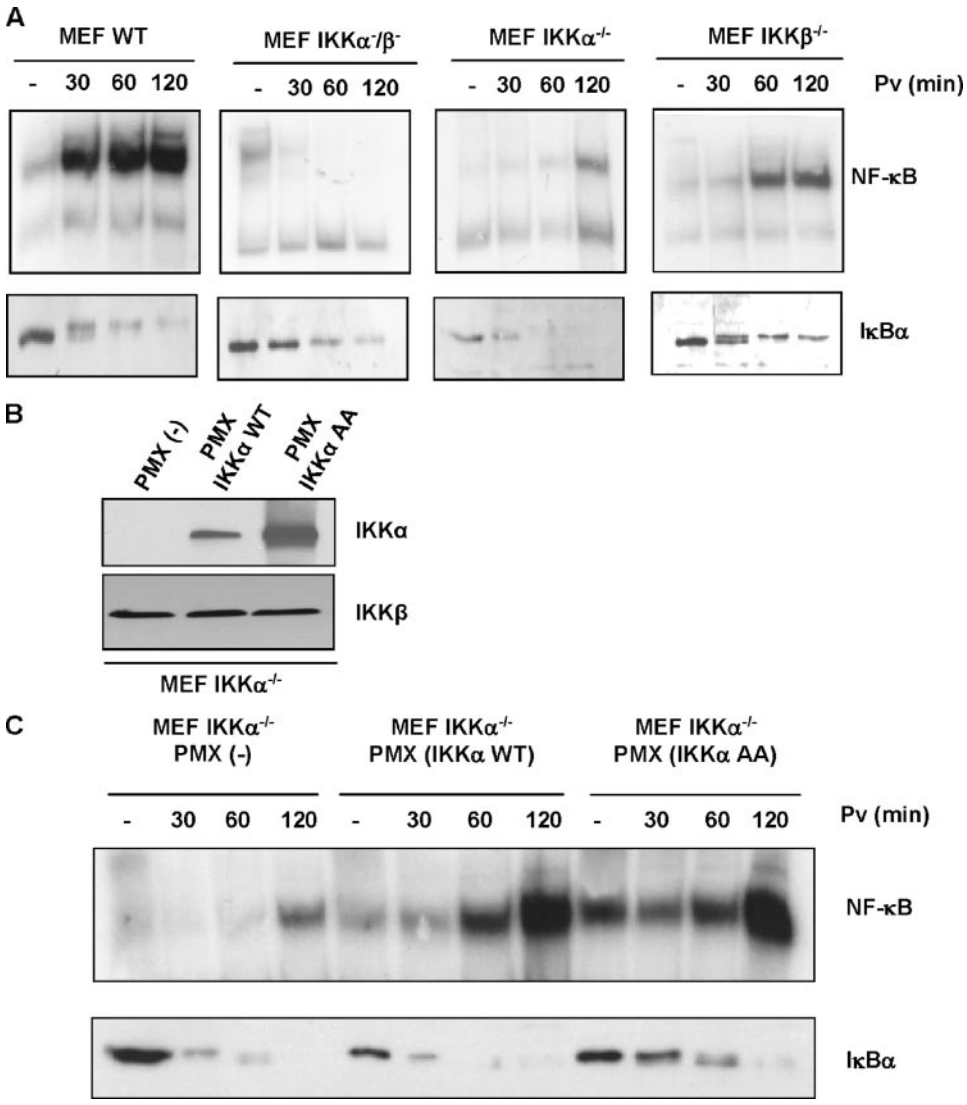

FIGURE 1. **IKKα is required for pervanadate-induced NF-κB DNA binding on an exogenous κB element.** *A*, WT MEFs or MEFs lacking IKKα/β, IKKα, or IKKβ were treated with sodium pervanadate (200 μM) for the indicated times. Nuclear extracts were analyzed by EMSA using a radioactive probe corresponding to the κB consensus sequence of the HIV-1 long terminal repeat, and IκBα degradation was detected by an anti-IκBα blot performed on cytoplasmic extracts. *B*, $ikk\alpha^{-/-}$ MEFs were infected with retrovirus containing either empty pMX vector or pMX encoding IKKα WT or AA. IKKα expression was checked by Western blotting. IKKβ Western blotting was also carried out as loading control. *C*, $ikk\alpha^{-/-}$ MEFs infected with retrovirus containing either empty pMX vector or pMX encoding IKKα WT or AA were treated with Pv. NF-κB activation was analyzed by EMSA with nuclear extracts, and IκBα degradation was detected by Western blotting with cytoplasmic extracts.

was carried out with saturated protein A-agarose beads. Cross-link was reversed at 65 °C for 4 h, and precipitated DNA was purified using phenol/chloroform extraction. Quantitative PCR (using SYBR green PCR master mix; Applied Biosystems) was performed on the immunoprecipitated DNA by normalizing to input DNA for each sample. The following primers, amplifying specific κB sites of the following genes, were used: *icam-1* forward, 5′-CATTACTTCAGTTTGGAAATTCCTAGATC3′; *icam-1* reverse, 5′-GGAACGAGGGCTTCGGTATT-3′; *mcp-1* forward, 5′-CACCCCATTACATCTCTTCCCC-3′; *mcp-1* reverse, 5′-TGTTTCCCTCTCACTTCACTCTGTC-3′; *IκBα* forward, 5′-TGGCGAGGTCTGACTGTTGTGG-3′; *IκBα* reverse, 5′-GCTCATCAAAAAGTTCCCTGTGC-3′.

RESULTS

IKKα Is Required for NF-κB DNA Binding on an Exogenous κB Element upon Pervanadate and TNF-α Stimulation—To assess potential additional roles of the IKK complex subunits beside the signal-induced degradation of the IκBα proteins in the cytoplasm, we treated MEF cells with sodium pervanadate (Pv). This treatment induces tyrosine phosphorylation and subsequent degradation of IκBα through a IKK-independent mechanism (12, 13, 29). As revealed by an electromobility shift assay using a probe corresponding to the κB site of the HIV-1 long terminal repeat, Pv induces a strong NF-κB activation in WT MEFs, due to an almost complete degradation of IκBα (Fig. 1*A*). Interestingly, Pv-induced NF-κB DNA binding activity is totally abolished in MEFs lacking both IKKα and IKKβ, despite unaltered IκBα degradation (Fig. 1*A*). The analysis of single knockout MEFs for either IKKα or IKKβ revealed that the abolition of NF-κB binding activity is still observable in $ikk\alpha^{-/-}$ MEFs, but there is a partial recovery of NF-κB DNA binding in $ikk\beta^{-/-}$ MEFs, suggesting that IKKα is required for NF-κB DNA binding on an exogenous κB element (Fig. 1*A*). To further confirm the role of IKKα in NF-κB binding, we reconstituted $ikk\alpha^{-/-}$ MEFs with retroviral expression vectors encoding either wild-type IKKα or the inactivable IKKα mutant harboring the mutations S176A and S180A within the activation loop (IKKα AA) (32). As control, we used $ikk\alpha^{-/-}$ MEFs reconstituted with an empty vector. Western blot analysis confirmed the expression of IKKα in reconstituted MEFs (Fig. 1*B*). We observed a recovery in NF-κB DNA binding in $ikk\alpha^{-/-}$ MEFs reconstituted with both IKKα WT and AA upon Pv stimulation but not in MEFs transduced with an empty vector (Fig. 1*C*). The kinetics of IκBα degradation were mostly the same in the three cell lines (Fig. 1*C*). These data suggest that IKKα contributes to the NF-κB DNA binding on an exogenous κB element in Pv-stimulated MEFs independently of its kinase activity. The same experiments were carried out with TNF-α as an inducer. Mobility shift assays revealed a clear inhibition of NF-κB DNA binding in $ikk\alpha^{-/-}$ MEFs transduced with an empty vector, com-

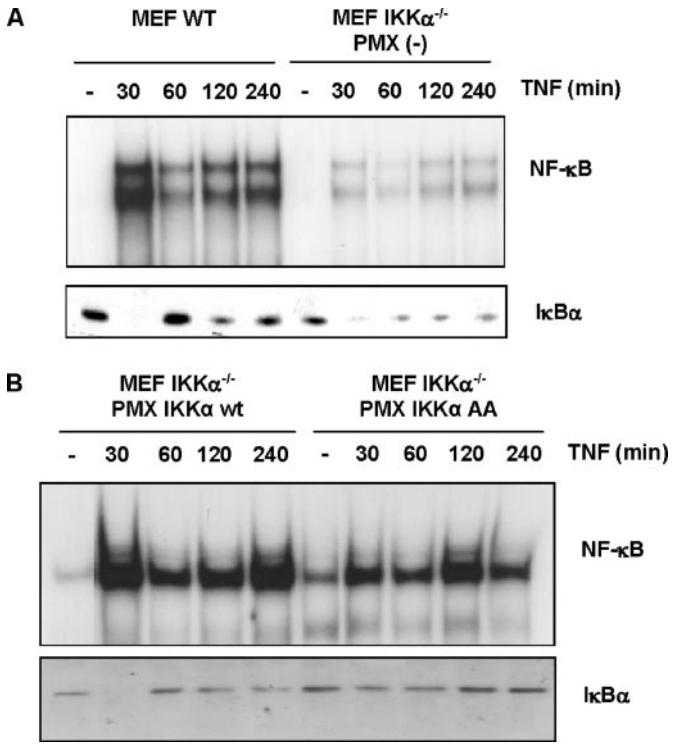

FIGURE 2. **IKKα is required for TNF-α-induced NF-κB DNA binding on an exogenous κB element.** *A* and *B*, MEFs that were WT and *ikkα*$^{-/-}$ transduced with an empty vector or a vector coding for IKKα WT or AA were treated with TNF-α (200 units/ml) for the indicated times. NF-κB activation and IκBα degradation were studied as described in the legend to Fig. 1.

pared with WT MEFs, despite a similar profile of IκBα degradation (Fig. 2*A*). Reconstitution of *ikkα*$^{-/-}$ MEFs with IKKα WT or AA restored a normal NF-κB binding upon TNF-α induction (Fig. 2*B*). Altogether, these results suggest that IKKα is required for NF-κB DNA binding independently of its kinase activity.

Mutation of IKKα Putative Nuclear Localization Signal Abrogates NF-κB DNA Binding in Vitro—Recently, a putative NLS was identified within the kinase domain of IKKα (33). To further explore whether IKKα must enter into the nucleus to modulate the NF-κB DNA binding activity, we generated a mutant form of IKKα bearing alanines instead of three important lysines within its NLS (33). This mutant failed to accumulate in the nucleus when overexpressed in 293T cells, whereas the wild-type IKKα did (Fig. 3*A*). Next we reconstituted *ikkα*$^{-/-}$ MEFs with retrovirus encoding IKKα NLS mutant. Western blot analysis confirmed the expression of IKKα NLS-mutated in reconstituted MEFs (Fig. 3*B*). To further characterize the functionality of IKKα NLS in MEFs, we carried out confocal microscopy. Detection of endogenous IKKα in WT MEFs revealed that the cellular distribution of IKKα is mainly cytoplasmic, but this protein also appears in the nucleus as *speckles* (Fig. 3*C*). Similar results were obtained using *ikkα*$^{-/-}$ MEFs transduced with WT IKKα (Fig. 3*C*). On the contrary, no nuclear distribution was observed using the NLS-mutated IKKα (Fig. 3*C*). Absence of immunoreactivity in *ikkα*$^{-/-}$ MEFs confirmed antibody specificity (Fig. 3*C*). This suggests that a small portion of IKKα enters within the nucleus in MEFs, and the mutation of IKKα NLS prevents this translocation. We next carried out a mobility shift experiment with MEFs expressing NLS-mutated IKKα compared with MEFs expressing WT IKKα. TNF-α and Pv-induced NF-κB DNA binding was strongly reduced in MEFs expressing NLS-mutated IKKα, despite a quite unaltered IκBα degradation (Fig. 3*D*). These data suggest that a small fraction of IKKα functions in the nucleus enhancing NF-κB DNA binding.

p65 Translocates Normally into the Nucleus in ikkα$^{-/-}$ *MEFs or MEFs Expressing NLS-mutated IKKα upon TNF-α Stimulation*—To explore whether the absence of NF-κB DNA binding in MEFs lacking IKKα or expressing IKKα NLS-mutated is due to an altered NF-κB nuclear translocation, we performed a large panel of anti-p65 Western blots on cytoplasmic and nuclear extracts of MEFs treated with TNF-α. As shown in Fig. 4*A*, p65 nuclear accumulation is maximal after 30 min of treatment in WT MEFs and then decreases at 60 min. The same profile of p65 nuclear translocation was observed in *ikkα*$^{-/-}$ MEFs reconstituted with an empty vector or expressing IKKα WT or AA (Fig. 4*A*). p65 nuclear translocation was also observed in MEFs where the IKKα NLS was mutated. Interestingly, the basal level of nuclear p65 is more important compared with other cells (Fig. 4*A*). Western blot analysis for cytoplasmic p65 revealed that its level of expression is constant across the analyzed cell lines (Fig. 4*A*). The purity of extracts was controlled by reprobing the membrane with antibodies raised against NBS and HSP60 (Fig. 4*A*). Unaltered p65 nuclear accumulation upon TNF-α stimulation of MEFs lacking IKKα or expressing IKKα NLS-mutated was further confirmed by immunolocalization of p65 using confocal microscopy (Fig. 4*B*). This experiment also confirmed an elevated basal nuclear accumulation of p65 in MEFs IKKα NLS-mutated (Fig. 4*B*). Altogether, we conclude that inhibition of NF-κB activation observed by EMSA in the absence of IKKα or when IKKα NLS is mutated is not due to a lack of p65 translocation.

IKKα Mediates Recruitment of p65 on Specific NF-κB-dependent Endogenous Promoters—To extend our analyses, we used p65 ChIP assays to determine whether defects in NF-κB DNA binding observed by electromobility shift assays were also confirmed on endogenous promoters. After chromatin immunoprecipitation with p65 antibody, real time PCR was used to amplify κB consensus sequences of promoters of three NF-κB target genes: *icam-1*, *mcp-1*, and *iκbα*. These genes were selected because they encode proteins important in inflammation and innate immunity (*icam-1* and *mcp-1*) or for the negative feedback of NF-κB regulation (*iκbα*). TNF-α treatment of WT MEFs induced two waves of p65 recruitment on *icam-1* and *mcp-1* promoter κB sites, which reached a maximum at 30 and 120 min. However, p65 recruitment is clearly inhibited in *ikkα*$^{-/-}$ MEFs, particularly after 30 and 60 min of treatment (Fig. 5, *A* and B). Interestingly, p65 recruitment on *iκbα* promoter in *ikkα*$^{-/-}$ MEFs is slightly reduced but not totally inhibited, as observed for *icam-1* and *mcp-1* (Fig. 5*C*). Similar results were obtained with the interleukin-6 promoter (data not shown). These data suggest that IKKα is required for p65 DNA binding on some but not all promoters. Reconstitution of *ikkα*$^{-/-}$ MEFs with a vector

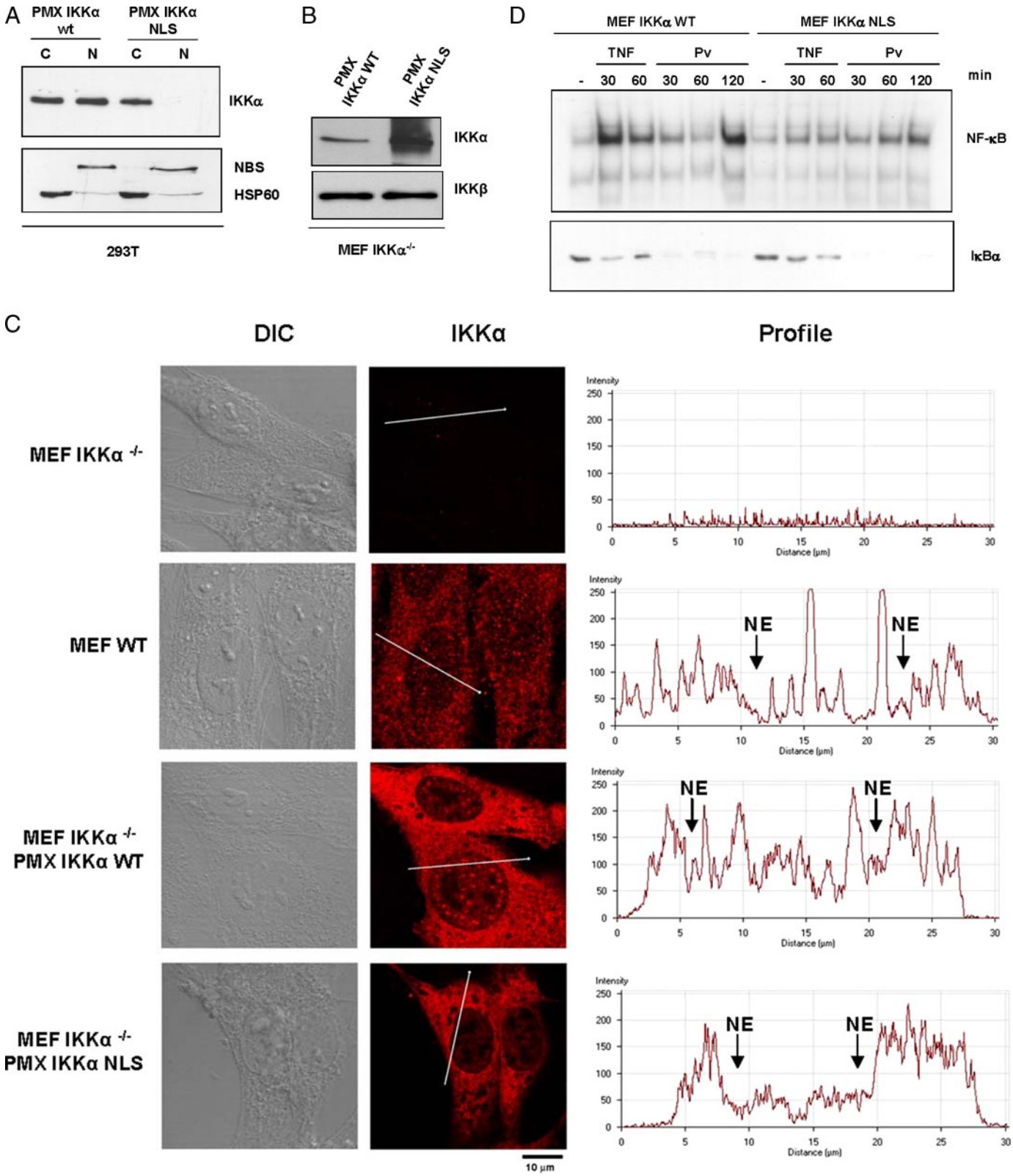

FIGURE 3. **Mutation of IKKα putative NLS abrogates IKKα nuclear accumulation and NF-κB DNA binding on an exogenous κB element.** *A*, pMX retroviral vector encoding IKKα wild-type or NLS-mutated was transfected into 293T cells. Nuclear and cytoplasmic extracts were then prepared, and cellular distribution of IKKα was analyzed by Western blotting. The purity of extracts was controlled by probing the membrane with antibodies raised against NBS and HSP60, a nuclear and mitochondrial protein, respectively. *B*, *ikkα*$^{-/-}$ MEFs were infected with retrovirus containing pMX vector encoding IKKα WT or NLS. IKKα expression was checked by Western blotting. IKKβ Western blotting was also carried out as loading control. *C*, IKKα cellular distribution was visualized by confocal microscopy in MEFs that were WT, *ikkα*$^{-/-}$, or *ikkα*$^{-/-}$ transduced with a vector coding for IKKα WT or NLS-mutated. Diagrams depict the intensity of the fluorescence for each cell type along *lines* drawn across the nucleus of the cells. *NE*, nuclear envelope. *D*, *ikkα*$^{-/-}$ MEFs transduced with a vector coding for IKKα WT or NLS-mutated were treated with TNF-α (200 units/ml) or Pv (200 μM) for the indicated times. NF-κB activation and IκBα degradation were studied as in Fig. 1.

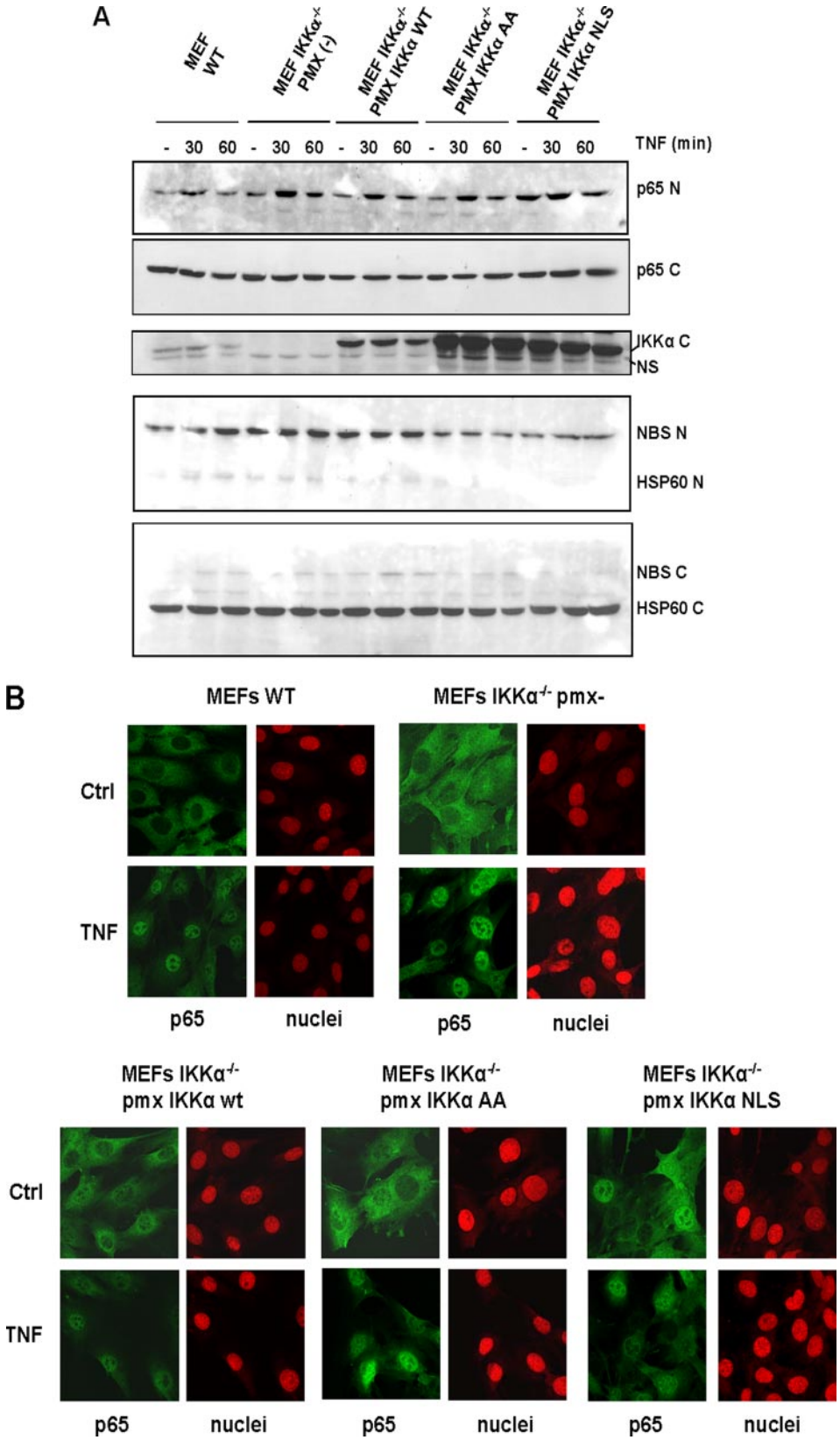

FIGURE 4. **p65 translocates normally into the nucleus upon TNF-α stimulation in MEFs lacking IKKα or expressing IKKα NLS-mutated.** *A*, MEFs that were wild-type or transduced with either an empty vector or vector coding for IKKα WT, AA, or NLS-mutated were treated with TNF-α (200 units/ml) for the indicated times. Nuclear (*N*) and cytoplasmic (*C*) extracts were prepared and analyzed by Western blotting using p65 and IKKα antibodies. The membrane was then stripped and reprobed with antibodies against HSP60 and NBS1 to check the purity of the extracts. *B*, MEFs that were wild-type or transduced with either an empty vector or vector coding for IKKα WT, AA, or NLS-mutated were treated with TNF-α for the indicated times. p65 immunofluorescence was then carried out as described under "Experimental Procedures" with a specific antibody (*green*). Nuclei were visualized using propidium iodide staining (*red*). *Ctrl*, control.

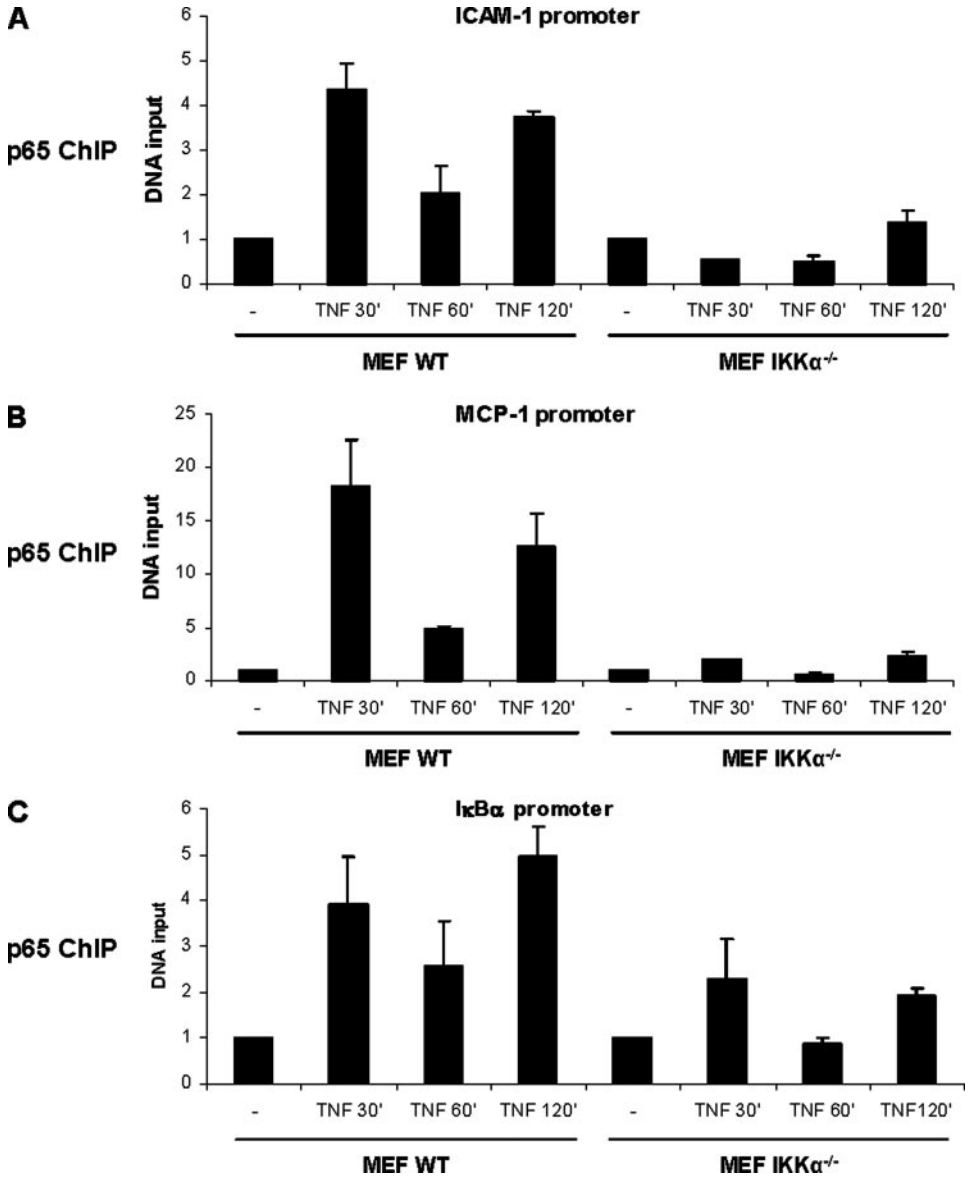

FIGURE 5. **IKKα mediates recruitment of p65 on specific NF-κB-dependent endogenous promoters.** MEFs that were wild-type or IKKα-deficient were treated with TNF-α for the indicated times. CHiP assays using a p65 antibody were performed as described under "Experimental Procedures." Real time PCR was then carried out on immunoprecipitated DNA using primers amplifying κB sites of promoters of *icam-1* (*A*), *mcp-1* (*B*), and *iκbα* (*C*) genes.

encoding WT IKKα completely restored recruitment of p65 on *icam-1* and *mcp-1* promoters, whereas no changes were observed regarding p65 DNA binding on *iκbα* promoter (Fig. 6, *A–C*). Complementation of *ikkα*$^{-/-}$ MEFs with IKKα AA or NLS clearly inhibits p65 recruitment on *icam-1* and *mcp-1* promoters but not on the *iκbα* promoter (Fig. 6, *A–C*). In this case, an increase in p65 DNA binding is even observed (Fig. 6*C*). Collectively, these experiments prompt us to conclude that IKKα is crucial for p65 DNA binding on some but not all promoters. Indeed, the *iκbα* promoter does not seem to require IKKα for p65 recruitment, whereas *icam-1* and *mcp-1* promoters do. p65 recruitment on these promoters is abrogated by IKKα AA or NLS mutations, whereas these mutations rather enhance p65 binding on the *iκbα* promoter.

Inhibition of p65 Binding on icam-1 and mcp-1 Promoters in ikkα$^{-/-}$ *MEFs Is Relieved by TSA Pretreatment*—Acetylation has been described as a critical post-translational mechanism regulating p65 DNA binding activity (34, 35), and treatment of cells with TSA, an HDAC inhibitor, potentiated NF-κB activation induced by TNF-α (36). This observation suggests that inhibition of HDAC activity is critical for a correct NF-κB DNA binding. Therefore, we wanted to explore whether TSA pretreatment modifies p65 DNA binding in *ikkα*$^{-/-}$ MEFs treated with TNF-α. TSA restored normal p65 recruitment on *icam-1* and *mcp-1* promoters in *ikkα*$^{-/-}$ MEFs (Fig. 7, *A* and *B*), reaching values similar to those obtained in WT MEFs (see Fig. 5). Conversely, TSA pretreatment did not increase p65 binding on the *iκbα* promoter (Fig. 7*C*). Altogether, these data suggest that TSA can substitute for IKKα for NF-κB DNA binding on specific promoters.

HDAC3 Recruitment on icam-1 and iκbα Promoters Is Oppositely Regulated by IKKα—The results obtained with TSA pretreatment led us to further explore the role of HDACs in IKKα-mediated p65 DNA binding. HDAC3, a class I histone deacetylase, has been reported to directly deacetylate p65, thereby inhibiting its DNA binding (34). HDAC3 is associated with NF-κB-dependent promoters and negatively regulates NF-κB-dependent transcription together with co-repressors, such as SMRT (20). Using laminin attachment to activate NF-κB, it was reported that IKKα directly removes SMRT and HDAC3 from promoters, thereby allowing NF-κB-mediated transcription (20). Thus, we studied the recruitment of HDAC3 on the promoters of two genes differentially regulated by IKKα (*i.e. icam-1* and *iκbα*) upon TNF-α stimulation. In *ikkα*$^{-/-}$ MEFs expressing WT IKKα, TNF-α induces a slight removal of HDAC3 from the *icam-1* promoter (Fig. 8*A*). On the contrary, HDAC3 is dramatically recruited in MEFS lacking IKKα and reconstituted with IKKα AA or NLS-mutated (Fig. 8*A*). No change in chromatin-bound HDAC3 was observed on the *iκbα* promoter in WT MEFs stimulated with TNF-α (Fig. 8*B*), whereas a dramatic removal is induced in *ikkα*$^{-/-}$ MEFs or MEFs expressing IKKα AA or NLS-mutated (Fig. 8*B*). This

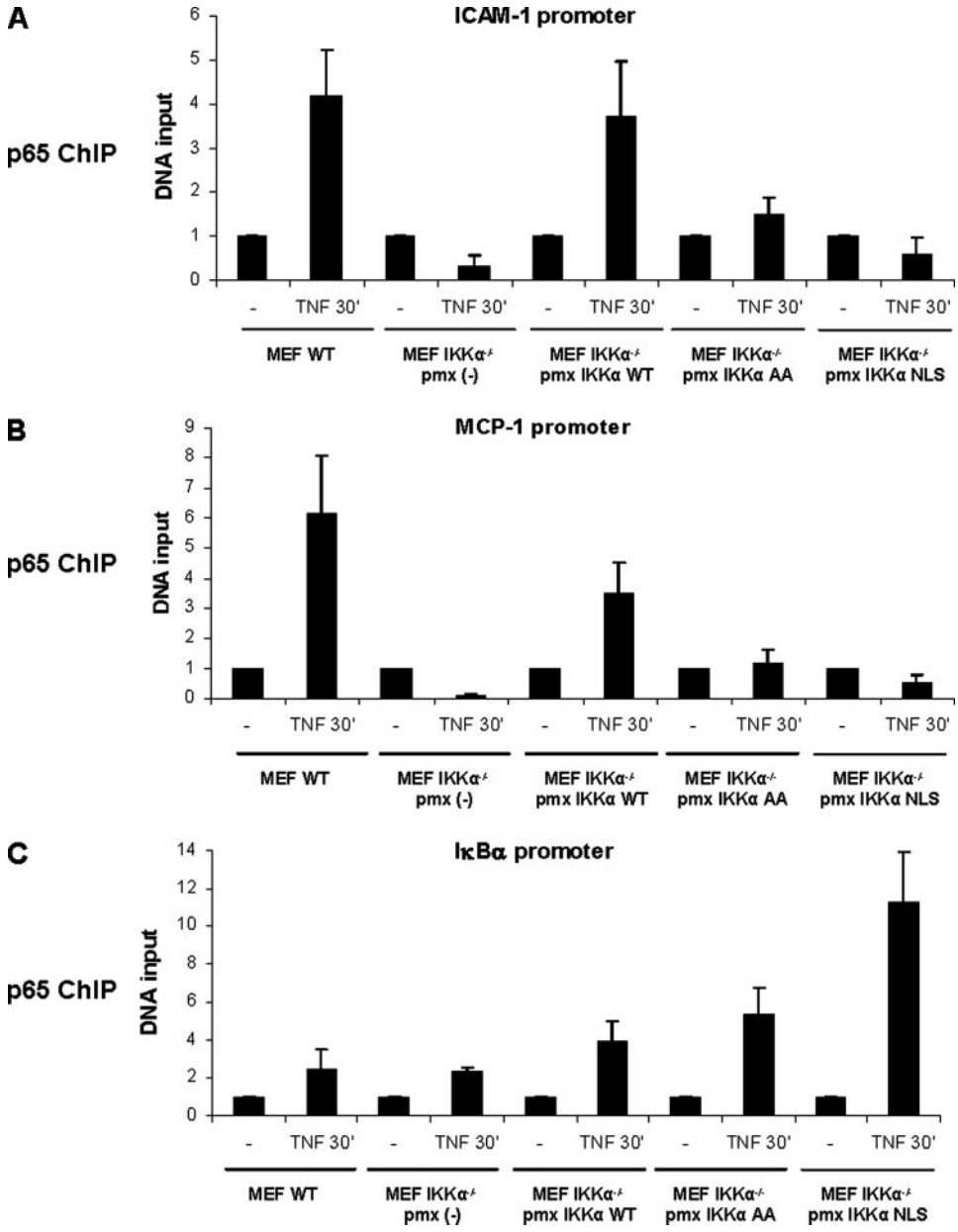

FIGURE 6. **p65 recruitment on specific endogenous promoters in MEFs expressing IKKα wild-type, AA, or NLS-mutated.** *ikkα*$^{-/-}$ MEFs transduced with either an empty vector or vector coding for IKKα WT, AA, or NLS-mutated were treated with TNF-α (200 units/ml) for the indicated times. CHiP assays using a p65 antibody were then performed as described in the legend to Fig. 5. *A*, *icam-1* promoter; *B*, *mcp-1* promoter; *C*, *iκbα* promoter.

HDAC3 removal is not induced by the prior p65 DNA binding, since it is still observable in p65 KO MEFs stimulated with TNF-α (data not shown). Collectively, these results suggest that IKKα oppositely regulates the dynamic of HDAC3 recruitment on *icam-1* and *iκbα* promoters.

DISCUSSION

The study of nuclear events involved in NF-κB activation is the focus of intensive research in many laboratories. IKKα, one of the subunits of the IKK complex, has recently been associated with NF-κB nuclear action, notably through its phosphorylation of histone H3, a component of nucleosomes (21, 23). However, the precise nuclear functions of IKKα are poorly understood. Here, the use of IKK-independent pathways triggering IκBα degradation (*i.e.* sodium pervanadate stimulation) allowed us to study the precise nuclear roles of IKK subunits in MEFs defective for IKKα and IKKβ. We found that IKKα is crucial for NF-κB DNA binding on an exogenous κB element, whereas IKKβ seems less important. TNF-α stimulation gave rise to the same results. This is in agreement with other studies demonstrating that the absence of IKKα inhibits NF-κB DNA binding activity in HeLa cells or mouse embryonic fibroblasts (25, 26). The complementation of *ikkα*$^{-/-}$ MEFs with either WT or inactivable (AA) IKKα totally rescued NF-κB DNA binding upon Pv and TNF-α stimulation, suggesting that IKKα kinase activity is not required for this function. Meanwhile, mutation of important residues in a putative NLS within IKKα, thereby preventing its nuclear translocation, dramatically inhibited NF-κB DNA binding on an exogenous κB element. This suggests that IKKα acts in the nucleus to enhance NF-κB DNA binding. Indeed, we clearly observed a nuclear distribution of IKKα as speckles in WT MEFs or *ikkα*$^{-/-}$ MEFs reexpressing IKKα WT, whereas mutation of IKKα NLS prevented this accumulation. We thus propose that a small portion of IKKα enters the nucleus to regulate chromatin events. This result seems to conflict with another work reporting an important nuclear accumulation of IKKα after TNF-α stimulation (21). We never observed such a massive translocation upon TNF-α treatment (data not shown). These discrepant results probably reflect technical differences between laboratories in the design of experiments. For example, the use of *ikkα*$^{-/-}$ cells to take into account aspecific binding of antibodies appears highly desirable to avoid artifactual results. In a more general context, IKKα nuclear translocation is still a matter of debate in literature. Whereas some researchers observe high levels of IKKα in the nucleus of various cell types (21, 23, 33, 37), others fail to detect any nuclear IKKα and even use this protein as a negative control in their ChIP experiments (38). These discrepancies probably reflect cell type-specific functions of IKKα.

Confocal microscopy experiments and p65 blots on cytoplasmic and nuclear fractions clearly demonstrated that the

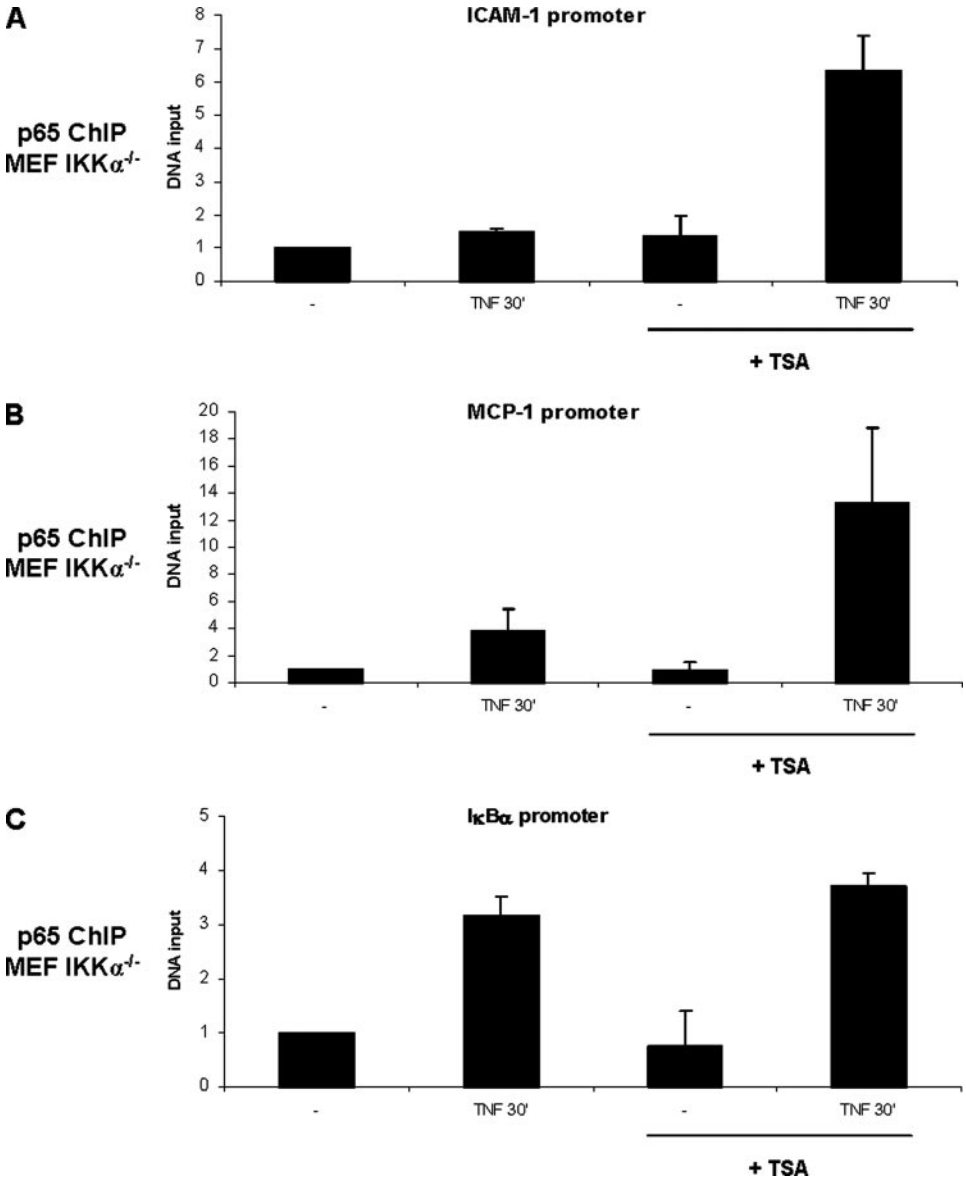

FIGURE 7. **Inhibition of p65 binding on *icam-1* (*A*), *mcp-1* (*B*), and *iκbα* (*C*) promoters in *ikkα*$^{-/-}$ MEFs is relieved by TSA pretreatment.** *ikkα*$^{-/-}$ MEFs were pretreated or not with TSA (450 μM, 2 h) and then stimulated with TNF-α (200 units/ml) for the indicated times. CHiP assays using a p65 antibody were then performed as described in the legend to Fig. 5.

absence of NF-κB DNA binding in MEFs defective for IKKα or expressing IKKα NLS-mutated was not due to an inhibition of p65 nuclear translocation. Interestingly, MEFs expressing IKKα NLS-mutated exhibit a constitutive nuclear accumulation of p65, suggesting that IKKα regulates p65 nucleo-cytoplasmic shuttling. Recently, Lawrence *et al.* (24) highlighted a role for IKKα in accelerating promoter clearance of p65 in macrophages, thereby contributing to the resolution of inflammation. This could explain the enhanced p65 nuclear accumulation observed in MEFs IKKα NLS-mutated, although, in this case, p65 is not found to be associated with target promoters.

In a second part, we extended our EMSA analyses to p65 ChIP assays. These experiments confirmed the dramatic decrease of p65 binding on *icam-1* and *mcp-1* promoters, two NF-κB target genes, in IKKα KO MEFs stimulated with TNF-α. On the contrary, the *iκbα* promoter does not seem to require IKKα for p65 binding, highlighting a promoter-specific function for IKKα. Normal p65 binding on the *iκbα* promoter in the absence of IKKα was also reported by other groups (21, 23), suggesting that this promoter behaves differently from *icam-1* and *mcp-1*. p65 ChIP also confirmed the crucial role of IKKα NLS sequence for p65 binding, suggesting that IKKα must enter into the nucleus to achieve its roles. Interestingly, IKKα catalytic activity seems to be also required for p65 DNA binding on endogenous *icam-1* and *mcp-1* promoters, whereas EMSA experiments revealed no change in NF-κB binding on an exogenous κB element using MEFs expressing IKKα AA. This suggests that IKKα has a dual function in p65 DNA binding. First, the requirement of nuclear IKKα for direct p65 binding on both exogenous κB elements and endogenous promoters suggests that IKKα directly modifies p65 to allow its DNA binding. This modification is likely to be acetylation, since IKKα has been recently reported to interact with CREB-binding protein, a histone acetyltransferase capable of acetylating p65 and thus enhancing its DNA binding (23, 34). IKKα might thus act as a nuclear scaffold protein required for the post-translational modifications of transcription factors, thereby enhancing their DNA binding capacities. The second role of IKKα involves its catalytic activity in the chromatin context. Hoberg *et al.* (20) recently reported that IKKα activity was necessary to phosphorylate the chromatin-bound corepressor SMRT, thereby inducing its removal together with HDAC3. This mechanism, deciphered upon NF-κB activation induced by laminin attachment, is likely to be the same in our system. Indeed, we observed a dramatic recruitment of HDAC3 on the *icam-1* promoter in MEFs lacking IKKα or expressing IKKα AA or NLS-mutated stimulated with TNF-α. One hypothesis is that promoter-bound HDAC3 inhibits p65 binding on the *icam-1* promoter by inducing its deacetylation, since this inhibition is relieved by TSA pretreatment. To further confirm this hypothesis, we transduced p65 KO MEFs with retroviruses encoding WT p65 or a hypoacetylated mutant containing a lysine to arginine substitution at the position 221, one major acetylation site of p65 (34). As already described, we found out that p65 K221R does not bind DNA due to a defect in nuclear translocation (data not shown). This

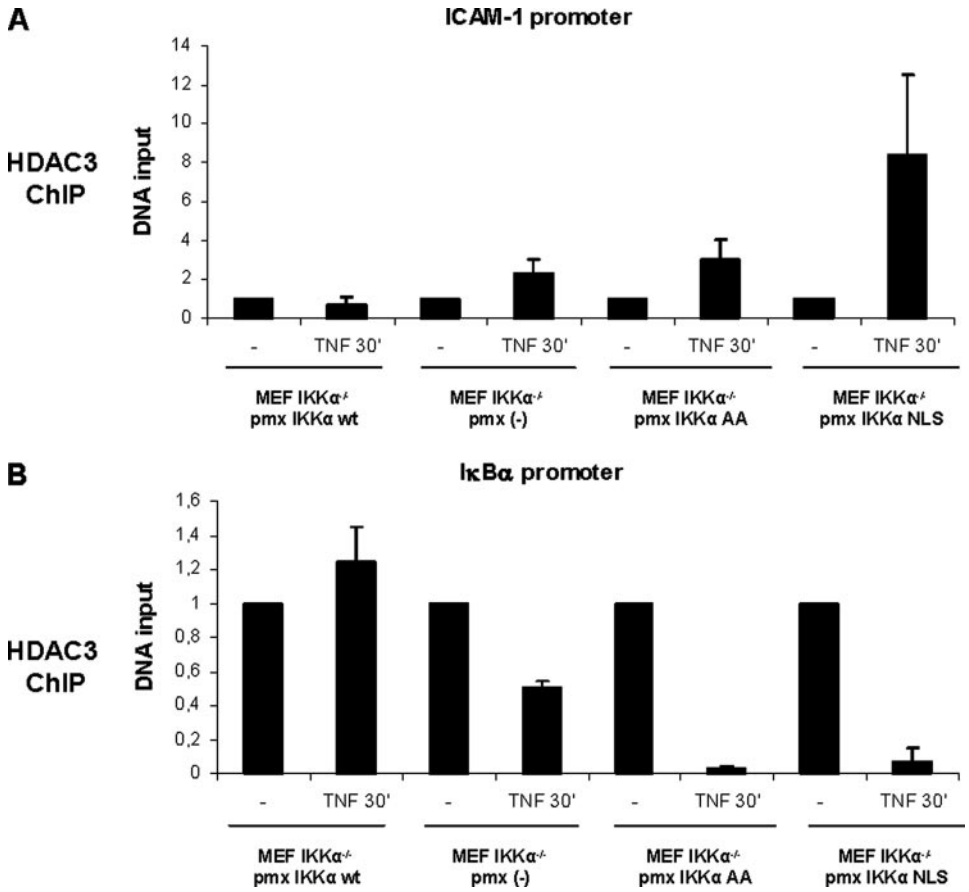

FIGURE 8. **HDAC3 recruitment on *icam-1* (*A*) and *iκbα* (*B*) promoters is oppositely regulated by IKKα.** *ikkα*$^{-/-}$ MEFs transduced with either an empty vector or vector coding for IKKα WT, AA, or NLS-mutated were treated with TNF-α (200 units/ml) for the indicated times. CHiP assays using a HDAC3 antibody were then performed as described in the legend to Fig. 5.

cytoplasmic retention is due to an enhanced interaction of the hypoacetylated p65 mutant with IκBα, which targets it to the cytoplasm in a manner dependent on the IκBα NES (34). Since the inhibition of p65 DNA binding observed in the absence of IKKα is not associated with a cytoplasmic sequestration, we can rule out at that time the possibility that IKKα targets p65 Lys^{221}. This suggests that another acetylation event, directly targeting p65 or not, is taking place.

An important finding in this work is that the *iκbα* promoter does not require IKKα for p65 DNA binding. Chromatin-bound HDAC3 is not removed from this promoter upon TNF-α stimulation, suggesting that the chromatin configuration of this promoter allows p65 binding without HDAC3 removal. A similar observation has been reported by another group (39). On the other hand, HDAC3 is removed from the *iκbα* promoter in IKKα KO MEFs or MEFs expressing IKKα AA or NLS-mutated, thereby inducing a greater p65 binding (at least for MEFs expressing IKKα AA or NLS-mutated). This suggests that IKKα would even function as a repressor that tethers small quantities of HDAC3 on the *iκbα* promoter, thereby preventing excessive p65 DNA binding. Collectively, our results strongly speak for a model in which the *iκbα* promoter behaves totally differently from other tested genes. These promoter specificities are poorly understood for the moment. Other works have reported that IKKα can phosphorylate histone H3, thereby modifying the transcriptional capacities of NF-κB (21, 23). We observed that histone H3 phosphorylation is inhibited on both *icam-1* and *iκbα* promoters in IKKα KO MEFs, ruling out the involvement of histone H3 in the binding of p65 (data not shown). Very recently, Huang *et al.* (40) have reported that IKKα also phosphorylates CREB-binding protein, which enhances its interaction with p65 and its histone acetyltransferase activity. In that context, it might be interesting to evaluate whether this phosphorylation is required in a promoter-specific context. In the same way, p65 binding on the *iκbα* promoter is largely unaffected by loss of glycogen synthase kinase 3β, whereas other promoters are glycogen synthase kinase 3β-dependent (41). As already proposed, this may be due to the presence of many NF-κB sites on this promoter that act synergistically to ensure DNA binding (41, 42). Additional work is necessary to determine the exact chromatin configuration of the *iκbα* gene and the regulators of IκBα expression. In conclusion, we demonstrated for the first time that IKKα mediates p65 DNA binding on specific promoters, which reconciles conflicting reports and highlights a new function for IKKα in NF-κB nuclear action. This gene specificity may help in the development of novel therapeutic strategies.

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