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IκB Kinase α and p65/RelA Contribute to Optimal Epidermal Growth Factor-induced c-*fos* Gene Expression Independent of IκBα Degradation*

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Mitogenic activation of expression of immediate-early genes, such as c-*fos*, is controlled through signal-induced phosphorylation of constitutively bound transcription factors that is correlated with a nucleosomal response that involves inducible chromatin modifications, such as histone phosphorylation and acetylation. Here we have explored a potential role for the transcription factor NF-κB and its associated signaling components in mediating induction of c-*fos* gene expression downstream of epidermal growth factor (EGF)-dependent signaling. Here we show that EGF treatment of quiescent fibroblast does not induce the classical pathway of NF-κB activation through IκB kinase (IKK)-directed IκBα phosphorylation. Interestingly, efficient induction of c-*fos* transcription requires IKKα, one of the subunits of the IκB kinase complex. The NF-κB subunit, p65/RelA, is found constitutively associated with the c-*fos* promoter, and knock-out of this transcription factor significantly reduces c-*fos* gene expression. Importantly, EGF induces the recruitment of IKKα to the c-*fos* promoter to regulate promoter-specific histone H3 Ser[10] phosphorylation in a manner that is independent of p65/RelA. Collectively, our data demonstrate that IKKα and p65/RelA contribute significantly to EGF-induced c-*fos* gene expression in a manner independent of the classical, IκBα degradation, p65/RelA nuclear accumulation response pathway.

NF-κB is a widely studied dimeric transcription factor that is inducible by inflammatory cytokines, lipopolysaccharide, and other regulatory stimuli (1–5). The five members of the mammalian NF-κB family are RelA/p65, c-Rel, p50/NF-κB1, p52/NF-κB2, and RelB. Control of NF-κB activation is generally considered to be at the level of release from the IκB inhibitory proteins, which maintain NF-κB largely in the cytoplasmic compartment. Following exposure of cells to inflammatory cytokines, the IκB kinase (IKK)[1] complex is activated and phosphorylates IκB proteins on N-terminal serines leading to their ubiquitination and subsequent proteasome-dependent degradation. This process frees NF-κB to accumulate in the nucleus and to associate with specific gene regulatory regions to control gene expression (1–5). The IKK complex contains two highly conserved catalytic subunits, namely IKKα and IKKβ, of which IKKβ appears to be the dominant kinase controlling IκB phosphorylation downstream of cytokine-induced signaling (1, 3). However little to no information exists regarding direct roles for IKK or NF-κB in controlling growth factor-induced gene expression.

EGF family members interact with receptors belonging to the EGF receptor family, including EGF receptor, epidermal growth factor receptor family protein (ErbB)2, ErbB3, and ErbB4 (6). Interaction of these signaling molecules with the receptors initiates intracellular signaling cascades that lead to activation of a number of transcription factors such as AP-1 and STATs (7). It has previously been reported that EGF induces NF-κB nuclear levels in cell types such as A431 cells and in several breast cancer cell lines that overexpress EGF receptor (8). However, it remains inconclusive whether EGF signaling to NF-κB occurs in EGF receptor-normalized cell lines such as fibroblasts, and relevant gene targets are unknown.

The immediate early genes (including c-*fos*, c-*jun*, and c-*myc*) are characterized by their rapid inducibility in response to a variety of signals, including mitogenic stimulation (9). c-*fos* has been the most extensively studied of this group of genes relative to induction of gene expression, with evidence demonstrating the involvement of sequence-specific transcription factors and associated co-factors controlling transcription induction in a manner dependent on the activation of upstream signal transduction cascades (10–12). A key regulatory element in the c-*fos* promoter is the serum response element (SRE), which interacts in a constitutive manner with the serum response ternary complex containing SRF, Elk-1, and the transcriptional co-activator CBP/p300 (10, 11, 13, 14). It has been demonstrated that phosphorylation of Elk-1 by MAPKs leads to the activation of the ternary complex-associated CBP/p300 histone acetyltransferase co-activator (14). Additionally, the c-*fos* promoter undergoes inducible histone H3 phosphorylation and acetylation following mitogenic stimulation that correlates with immediate-early gene induction (15–17). The kinase that controls histone H3 phosphorylation downstream of mitogen-induced signaling has been proposed to be either MSK1/2 or ribosomal S6 Kinase 2 (18, 19). More recent evidence supports the role of MSK1/2 in controlling this response (18). Recently we and others (20, 21) described IKKα as a critical kinase

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[1] The abbreviations used are: IKK, IκB kinase; EGF, epidermal growth factor; STAT, signal transducers and activators of transcription; SRE, serum response element; CBP, cAMP-responsive element-binding protein-binding protein; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; MEF, mouse embryonic fibroblast; ChIP, chromatin immunoprecipitation; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SRF, serum response factor; MSK, mitogen- and stress-activated protein kinase.

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involved in controlling TNF-induced H3 Ser^{10} phosphorylation at certain NF-κB-dependent promoters in a manner dependent on the recruitment of RelA/p65 to the promoters. Here we have analyzed the potential involvement of the p65/RelA subunit of NF-κB and IKKα in controlling EGF-induced c-*fos* gene expression.

EXPERIMENTAL PROCEDURES

Cells and Reagents—IKK wild-type, $IKK\alpha^{-/-}$, and $IKK\beta^{-/-}$ MEFs were provided by I. Verma and M. Karin. IKKα reconstituted MEFs ($IKK\alpha^{+/+}$) were provided by E. Dejardin and D. Green. Antibodies against IκBα, CBP, tubulin, and mouse IgG were obtained from Santa Cruz. The p65/RelA-specific antibody was obtained from Rockland, Inc. Antibodies specific for IKKα, phospho-Ser^{10}-H3 and acetyl-H3 at Lys^9 or Lys^{14} were obtained from Upstate Biotechnology Inc. Phospho-MEK1/2 and phospho-Elk-1 antibodies were obtained from Cell Signaling. EGF (Upstate Biotechnology Inc.) was used at a final concentration of 50 ng/ml.

Chromatin Immunoprecipitation Assay—ChIP analysis was performed following a protocol provided by Upstate Biotechnology, Inc. under modified conditions and as previously described (20). The following promoter-specific primers were used; primer pair CACGGCCGGTC-CCTGTTGTTC and GTCGCGGTTGGAGTAGTAGGCG was used to amplify the proximal region of the c-*fos* promoter. The primers for β-globin are CAGCATGTGCTGAGGACTTGG and ACTGCCTTCA-GAGAATCGCCC. Quantitative real time PCR was performed in triplicate to determine the association of phospho-Ser^{10}-H3, acetyl-Lys^9-H3, and acetyl-Lys^{14} with the c-*fos* promoter by using the above oligonucleotide primers and input DNA standards diluted in 3-fold increment from 10 to 0.01% with SYBR Green Master Mix and the ABI Prism 7700 sequence detection system.

Quantitative Real Time PCR—Total RNA was prepared from MEF, $IKK\alpha^{-/-}$, $IKK\alpha^{+/+}$, $IKK\beta^{-/-}$, or $p65^{-/-}$ cells using TRIzol (Invitrogen) as recommended by the manufacturer. For real time PCR, the cDNA was prepared with random primers (Invitrogen) and analyzed in triplicate with the SYBR Green Master Mix (Qiagen) for 15 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 30 s and 63 °C for 30 s in the ABI sequence detection system. The oligonucleotide primers used to analyzed *IκBα* transcripts contained the sequences 5′-GATC-CGCCAGGTGAAGGG-3′ and 5′-GCAATTTCTGGCTGGTTGG-3′. Primers used to analyze the c-*fos* and 18 S ribosomal RNAs were obtained using an Ambion gene-specific relative reverse transcription-PCR kit with the suggested conditions.

Western Blot Analysis—Western blot analysis was performed after preparing nuclear, cytoplasmic, or whole cell extracts and separating proteins by SDS-PAGE followed by immunoblotting with antibodies as indicated. Extractions of acid-soluble proteins were done according to the protocol described by Upstate Biotechnology, Inc. and resolved on 10–20% Tris-Tricine SDS-PAGE gels.

RESULTS

Analysis of the Classical NF-κB Activation Pathway in Response to EGF Stimulation of MEFs—It has previously been reported that EGF induces NF-κB nuclear levels in cell types that overexpress EGF receptor, such as A431 cells and certain breast cancer cell lines (8). We sought to determine whether EGF signaling activates the classical NF-κB pathway in cells that do not express high levels of the EGF receptor. Thus, EGF-treated mouse embryonic fibroblasts were analyzed for the characteristic degradation of IκBα and nuclear accumulation of the p65 NF-κB subunit typically associated with the TNF response. MEFs were stimulated with EGF and assayed for degradation of IκBα through Western blot analysis. Interestingly, EGF did not result in any significant level of IκBα degradation (Fig. 1*A*). Additionally, analysis of nuclear and cytoplasmic extracts from EGF-treated MEFs did not reveal detectable nuclear translocation of p65 (Fig. 1*B*), although similar analysis using TNF results in strong IκBα degradation and p65 nuclear accumulation (Ref. 20 and data not shown). This is consistent with previous reports indicating that maximal nuclear accumulation of p65 is largely dependent on IKK-directed IκBα phosphorylation. We previously reported that the subcellular localization of IKKα in unstimulated MEFs is both nuclear and cytoplasmic and that IKKα

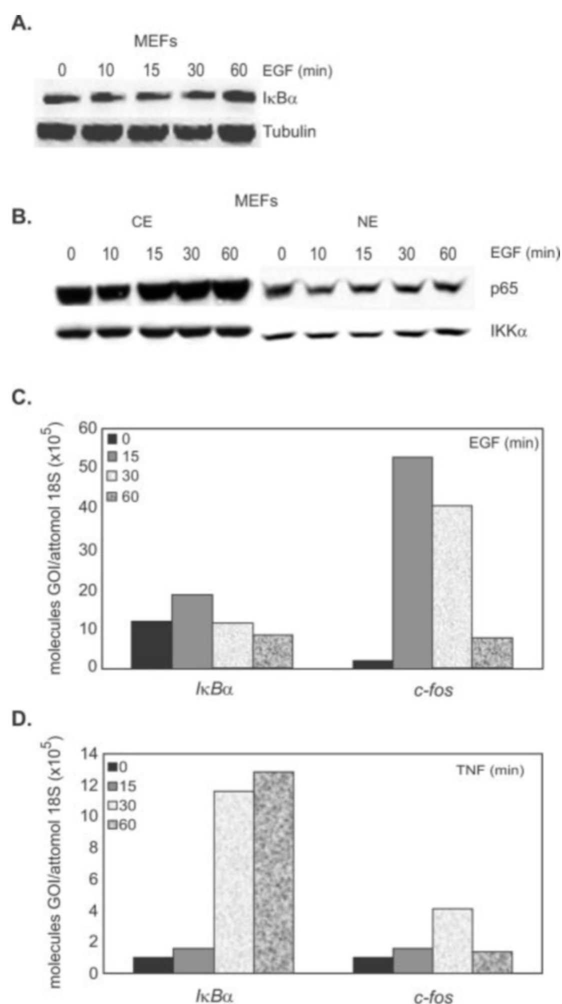

FIG. 1. **EGF does not activate the classical NF-κB activation pathway in MEFs.** *A*, detection of IκBα protein levels in EGF-stimulated IKK wild-type MEFs. The cells were serum-starved for 48 h and treated with EGF (50 ng/ml) at the indicated time points. Western analysis was performed with anti-IκBα (*top panel*) and anti-tubulin (*bottom panel*) as a loading control. *B*, analysis of the subcellular localization of p65 NF-κB subunit and IKKα in EGF-treated MEFs. MEFs were stimulated with EGF as in *A*, fractionated into cytoplasmic (*CE*) and nuclear (*NE*) fractions, and then analyzed directly by Western blotting with IKKα and p65 antibodies. *C*, real time PCR analysis was performed to measure the endogenous levels of *IκBα* or c-*fos* mRNA in EGF (50 ng/ml) stimulated IKK wild-type MEFs. Quiescent cells were treated with EGF at the indicated time points. The values are reported as molecules of gene of interest (*GOI*), *IκBα* or c-*fos*, per attomol of 18 S rRNA copies. *D*, real time PCR analysis of endogenous levels of *IkBα* or c-*fos* mRNA in TNF-treated (10 ng/ml) quiescent IKK wild-type MEFs.

undergoes a nuclear translocation event in response to TNFα stimulation (20). To determine whether EGF induces a similar response, IKKα levels were examined in nuclear and cytoplasmic extracts by Western analysis. Similar to p65, nuclear levels of IKKα remained unchanged in response to EGF (Fig. 1*B*). NF-κB DNA binding potential, analyzed by electrophoretic mobility shift assay, did not show any appreciable increase in response to EGF (data not shown). Additionally, real time PCR analysis of wild-type MEFs demonstrated only a modest induction of expression of the NF-κB-dependent *IκBα* gene in response to EGF in contrast to the stronger inducible profile observed for induction of c-*fos* gene expression (Fig. 1*C*). In contrast, TNF strongly activated the *IκBα* gene and only weakly induced c-*fos* gene expression (Fig. 1*D*), as previously reported (22). Taken together, these results demonstrate that EGF does not activate the classical NF-κB activation pathway in mouse embryonic fibroblasts.

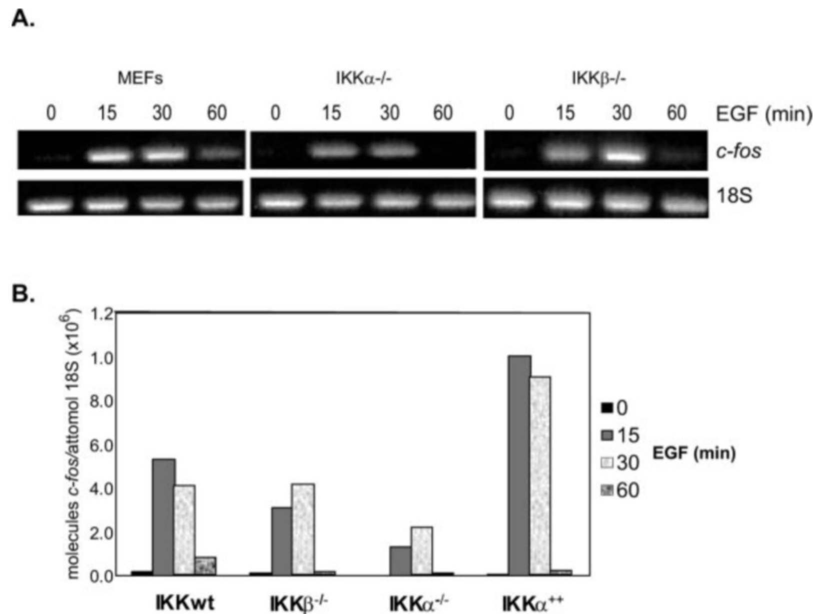

FIG. 2. **IKKα is required for optimal EGF-induced expression of c-*fos*.** *A*, reverse transcription-PCR analysis was performed to measure endogenous mRNA levels of c-*fos* after EGF stimulation. Quiescent IKK wild-type (*left panel*), IKKα $^{-/-}$ (*middle panel*), or IKKβ$^{-/-}$ MEFs were treated with EGF (50 ng/ml) at the indicated time points and then harvested. Levels of 18 S rRNA were also measured as a control. *B*, real time PCR analysis was performed to quantitate the endogenous levels of c-*fos* mRNA. Quiescent IKK wild-type, IKKβ$^{-/-}$, IKKα$^{-/-}$, or IKKα$^{+/+}$ reconstituted MEFs were treated with EGF at the indicated time points, similar to *A*. The values are reported as molecules of c-*fos* per attomol of 18 S rRNA copies.

IKKα Is Required for Optimal EGF-induced c-fos Gene Expression—Previously, we had found that IKKα contributes to cytokine-induced gene expression. Thus, we addressed whether the loss of IKKα has an observable affect on c-*fos* gene expression in response to EGF. Analysis of c-*fos* gene expression was measured by reverse transcription-PCR (Fig. 2*A*) and independently by quantitative real time PCR analysis (Fig. 2*B*) in wild-type MEFs and MEFs deficient for either IKKα or IKKβ. MEFs were maintained in low serum (0.5% fetal bovine serum) for 48 h followed by EGF stimulation for the indicated times to induce c-*fos* gene expression. As expected, wild-type MEFs exhibited rapid induction of c-*fos* mRNA with peak levels occurring 15 min after EGF stimulation, which decreased by the 1-h time point (Fig. 2). Interestingly, induction of c-*fos* in IKKα$^{-/-}$ MEFs was considerably reduced and more transient following EGF stimulation (Fig. 2). Although induction of c-*fos* was reduced at the 15-min time point in IKKβ$^{-/-}$ cells, the levels were more comparable with wild-type cells following 30 min of EGF stimulation (Fig. 2). Restoration of IKKα-deficient cells with wild-type IKKα (IKKα$^{+/+}$) resulted in strong c-*fos* expression following EGF treatment (Fig. 2*B*). The enhanced level of c-*fos* gene expression in the IKKα$^{+/+}$ MEFs is likely due to the relatively high expression of reconstituted IKKα in these cells (data not shown). Additionally, a similar requirement for IKKα in the inducible regulation of two other immediate-early genes, c-*myc* and *Fra-2*, was observed (data not shown). These data provide evidence that IKKα is required for optimal EGF-induced c-*fos* gene expression.

The Loss of IKKα Does Not Affect EGF-induced Signaling Pathways—One possible mechanism for the reduction of c-*fos* gene expression observed in the IKKα$^{-/-}$ MEFs may involve inhibition of EGF-induced signaling pathways. Therefore, we investigated the effect of EGF on MAPK activation in IKKα-deficient cells by Western blot analysis using phospho-specific antibodies. Treatment of wild-type MEFs with EGF resulted in the induction of MEK1/2 phosphorylation within 10 min and decreased after 30 min (Fig. 3*A*). Induction of MEK1/2 phosphorylation in IKKα$^{-/-}$ MEFs was similar to wild-type MEFs with slight kinetic differences in activation (Fig. 3*A*). MAPK-directed phosphorylation of Elk-1 was analyzed by immunoblotting with a phospho-specific Elk-1 antibody. Inducible and transient levels of Elk-1 phosphorylation were detected in both wild-type and IKKα-deficient MEFs (Fig. 3*B*). These results indicate no significant defect in MAPK activation in IKKα$^{-/-}$ cells and suggest that the altered induction in c-*fos* gene expression in IKKα$^{-/-}$ cells is downstream of MAPK activation, specifically at the level of c-*fos* gene regulation.

Recruitment of IKKα to the Endogenous c-fos Promoter Is Enhanced in Response to EGF—Prior studies have correlated enhanced H3 Ser^{10} phosphorylation with transcriptional induction of immediate-early genes, such as c-*fos* and c-*myc*, upon mitogenic treatment (15–17, 19). However, the promoter association of potential H3 Ser^{10} kinases has not been explored. Thus, we investigated whether IKKα is recruited to the c-*fos* promoter in response to EGF in a manner associated with specific histone modifications. Specifically, MEFs were maintained for 48 h in 0.5% serum. Quiescent cells were treated with EGF (50 ng/ml) for various times, DNA and protein was cross-linked with formaldehyde, and DNA was sonicated to shear chromatin into average fragments of ~1 kb. Subsequently, protein-DNA complexes were immunoprecipitated using antibodies that specifically recognize phospho-Ser^{10} histone H3, acetyl-Lys^{9}-H3, or acetyl-Lys^{14}-H3 in parallel with an IKKα-specific antibody. DNA recovered from the antibody-bound fractions as well as the DNA from input chromatin (input) were analyzed by semi-quantitative PCR using primers specific for the proximal region of the c-*fos* promoter. The specificity for all ChIP assays was demonstrated both by the absence of c-*fos* amplification in the mock samples immunoprecipitated with or without an irrelevant antibody, IgG, and by analysis of the transcriptionally inactive β-globin locus (data not shown).

In wild-type MEFs, stimulation with EGF led to an increase of H3 Ser^{10} phosphorylation at the c-*fos* promoter, with peak levels occurring at the 10-min time point that declined to unstimulated levels 1 h following EGF stimulation (Fig. 4*A*, *left panel*). In contrast to H3 Ser^{10} phosphorylation, acetylation of H3 at Lys^{9} and Lys^{14} was detected in unstimulated cells with a modest increase detected following EGF stimulation. Interestingly, association of IKKα with the c-*fos* promoter was detectable at low levels in unstimulated cells and increased transiently in response to EGF, with enhanced association occurring after 10 min of EGF treatment (Fig. 4*A*, *left panel*). This analysis demonstrates that IKKα association with the c-*fos* promoter correlates with induced H3 Ser^{10} phosphorylation levels in quiescent MEFs stimulated with EGF.

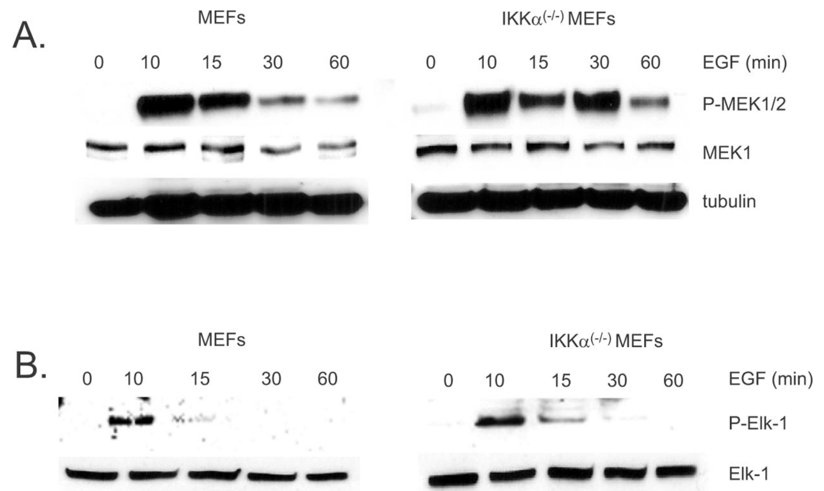

FIG. 3. **EGF-induced MAPK activation is unaffected in IKKα$^{-/-}$ MEFs.** Analysis of MAPK activation in EGF-stimulated IKK wild-type and IKKα$^{-/-}$ MEFs. The cells were serum-starved for 48 h and treated with EGF (50 ng/ml) at indicated time points. Western analysis was performed with anti-phospho-MEK1/2 (*A*, *top panel*) and antibodies against MEK1 and tubulin (*A*, *bottom panel*) as a loading control. *B*, kinetics of EGF- induced Elk-1 phosphorylation levels in IKK wild-type and IKKα$^{-/-}$ MEFs were analyzed by Western with antibodies against phospho-Elk-1(*top panel*) and total Elk-1 (*bottom panel*).

IKKα Is Required for EGF-induced H3 Ser^{10} Phosphorylation at the c-fos Promoter—To determine whether IKKα is required for EGF-stimulated H3 Ser^{10} phosphorylation at the c-*fos* promoter, ChIP assays were performed using quiescent IKKα$^{-/-}$ MEFs. In contrast to wild-type MEFs (Fig. 4*A*, *left panel*), enrichment for H3 Ser^{10} phosphorylation at the c-*fos* promoter was undetected in IKKα$^{-/-}$ MEFs following EGF treatment (Fig. 4*A*, *middle panel*). Additionally, the kinetic profiles of Lys^{9}- and Lys^{14}-acetylated H3 were similar to wild-type cells; however, reduced levels of acetylated H3 at Lys^{19} or Lys^{14} were detected in IKKα$^{-/-}$ cells. Next, quantification of ChIP DNA by real time PCR was performed to measure the amount of c-*fos* genomic DNA that co-immunoprecipitated with the specific histone modifications in wild-type and IKKα$^{-/-}$ cells relative to total input chromatin. These results show that in IKKα$^{-/-}$ MEFs, H3 Ser^{10} phosphorylation levels are quantitatively reduced most significantly after 10 min of EGF treatment with more comparable levels to wild type at later time points (Fig. 4*B*). Both Lys^{9}- and Lys^{14}-acetylated H3 show modest induction in response to EGF in wild-type MEFs. However, induction and overall levels of acetylated H3 are reduced in IKKα$^{-/-}$ MEFs (Figs. 4, *C* and *D*).

Next, to more directly address a potential requirement for IKKα in modulating H3 Ser^{10} phosphorylation at the c-*fos* promoter, ChIP assays were performed using IKKα-null MEFs where wild-type IKKα was restored via stable expression (indicated as IKKα$^{+/+}$). ChIP assays revealed that H3 Ser^{10} phosphorylation levels were restored in response to EGF in IKKα$^{+/+}$ MEFs with slight differences in kinetics as compared with wild-type cells, with peak levels occurring after 15 min of EGF treatment (Fig. 4*A*, *right panel*). The kinetic profile of IKKα recruitment was delayed as compared with wild type with peak levels of promoter-associated IKKα detected after 15 min of EGF treatment. Additionally, the kinetic profiles for Lys^{9}- and Lys^{14}-acetylated H3 were comparable with wild-type MEFs. Overall, the recruitment of IKKα at the c-*fos* promoter coincided with and was required for promoter-associated H3 Ser^{10} phosphorylation following EGF treatment.

IKKα Controls Global Levels of EGF-induced Phosphorylation of Histone H3 on Ser^{10}—To address whether IKKα plays a broader role in regulating global levels of histone H3 phosphorylation, core histones were acid-extracted from EGF-treated wild-type MEFs or IKKα-deficient cells (IKKα$^{-/-}$) and examined by Western blot analysis using an antibody specific to phospho-histone H3 (Ser^{10}). As expected, wild-type MEFs exhibit inducible H3 Ser^{10} phosphorylation in response to EGF in a time-dependent manner (Fig. 4*E*). This histone modification was visible within 15 min after the addition of EGF with overall levels decreasing between 30 and 60 min post-induction. Although H3 Ser^{10} phosphorylation levels in IKKα$^{-/-}$ MEFs were similar to wild type after 15 min of EGF treatment, overall levels were lost at the later time points (Fig. 4*E*). IKKα$^{+/+}$ MEFs, in which functional IKKα was stably expressed in IKKα$^{-/-}$ cells, result in restored levels of phosphorylated Ser^{10} levels with similar kinetics as wild-type MEFs (Fig. 4*E*). Global levels of EGF-induced histone H3 acetylation at Lys^{9} and Lys^{14} were slightly reduced in IKKα$^{-/-}$ cells as compared with wild-type cells (Fig. 4*E*). These data support a role for IKKα in modulating global levels of histone H3 phosphorylation on Ser^{10} in response to mitogenic (EGF) stimuli.

p65/RelA Is Constitutively Associated with the c-fos Promoter and Contributes to the Induction of c-fos Gene Expression—Transcriptional regulation of c-*fos* is controlled by *cis*-acting elements in the promoter regions, including the SRE and the 12-*O*-tetradecanoylphorbol-13-acetate response element (see Ref. 23 and references therein). Full activation of the c-*fos* SRE requires association with the ubiquitous transcription factor SRF and formation of a complex with ternary complex factors, including the Ets family of transcription factor Elk-1 (10). Additionally, transactivation by these nuclear factors is facilitated by CBP/p300 (14). In terms of NF-κB-dependent promoter assembly, previous reports have demonstrated that IKKα interacts with CBP; however, recruitment of CBP to NF-κB-dependent promoters is not dependent on IKKα (21). In addition, utilizing p65$^{-/-}$ MEFs, the p65 subunit was shown to be required for IKKα association with NF-κB-regulated promoters following TNF stimulation (20, 21). To explore the mode of recruitment for IKKα to the c-*fos* promoter, ChIP assays were performed using antibodies against p65 and CBP. In wild-type MEFs, p65 was detected at the c-*fos* promoter in unstimulated cells, and this remained relatively unchanged following EGF stimulation (Fig. 5*A*, *left panel*). A similar profile of p65 association with the c-*fos* promoter was observed in IKKα$^{-/-}$ MEFs (Fig. 5*A*, *right panel*). Consistent with previous reports (see Ref. 14 and references therein); CBP is found constitutively associated with the c-*fos* promoter in wild-type MEFs (Fig. 5*A*, *left panel*). Additionally, CBP recruitment was unaltered in cells deficient for IKKα (Fig. 5*A*, *right panel*). p65 was not found associated with the β-globin promoter (data not shown), indicating the specificity of the ChIP assays. These data indicate that p65 is preassociated with the c-*fos* promoter in an IKKα-independent manner.

To explore a potential role for p65 at the c-*fos* gene promoter, quantitative real time PCR analysis was used to measure c-*fos*

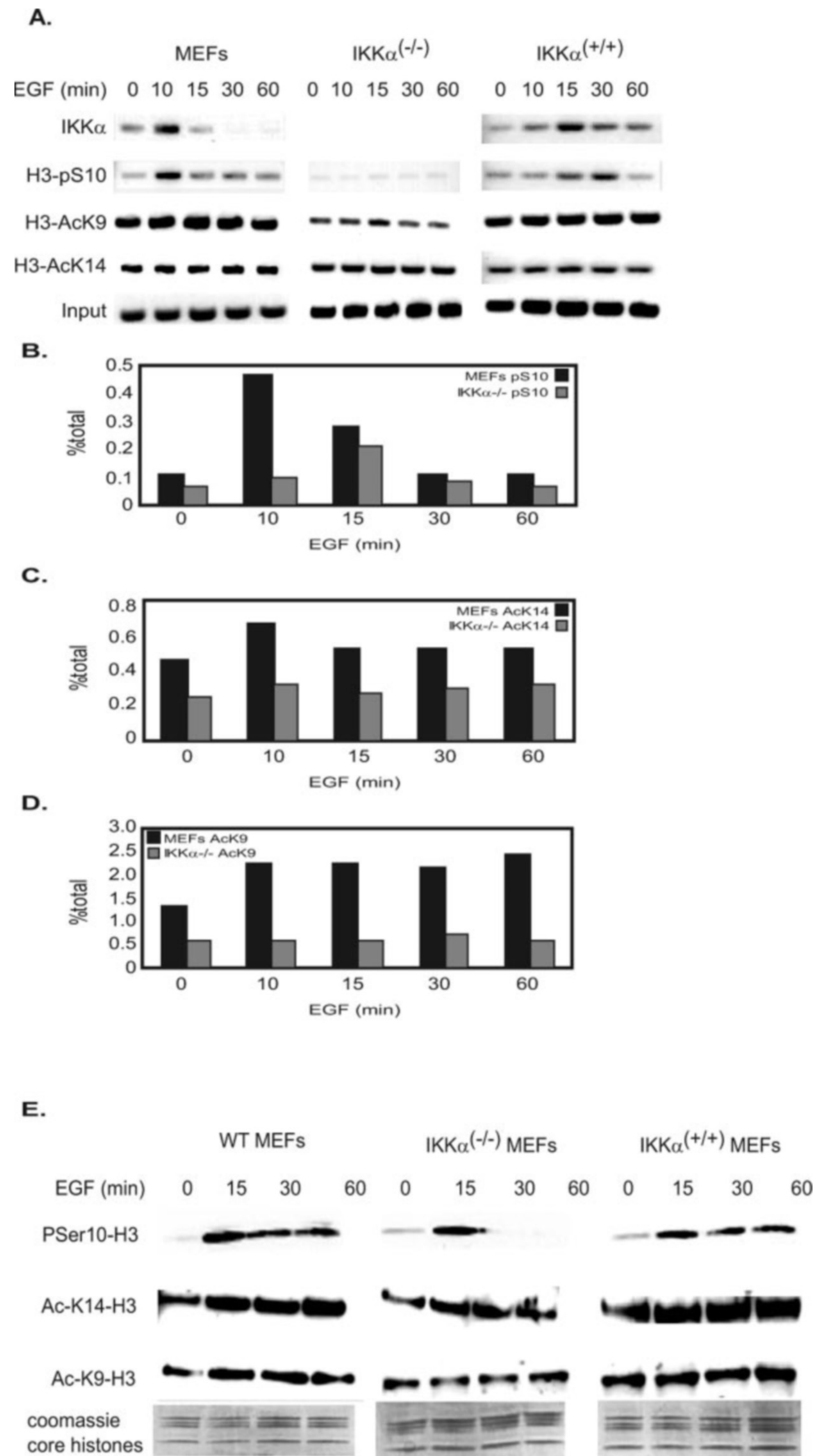

FIG. 4. **EGF-induced H3 Ser^{10} phosphorylation at the c-*fos* promoter requires IKKα recruitment.** *A*, chromatin was prepared from quiescent IKK wild-type (*left panel*), IKK$\alpha^{-/-}$ (*middle panel*), or IKK$\alpha^{+/+}$, IKKα reconstituted MEFs (*right panel*) that were treated with EGF (50 ng/ml) at the indicated time points. ChIP assays were performed using anti-IKKα, anti-phospho-Ser^{10} (*H3-pS10*), anti-acetyl-Lys^9 (*H3-AcK9*), or anti-acetyl-Lys^{14} (*H3-AcK14*) as indicated. c-*fos* promoter DNA sequences were detected by semi-quantitative PCR. *B–D*, chromatin was prepared from quiescent IKK wild-type (*black bars*) or IKK$\alpha^{-/-}$ (*gray bars*) MEFs that were treated with EGF (50 ng/ml) at the indicated time points. ChIP assays were performed using anti-phospho-Ser^{10} (*B*), anti-acetyl-Lys^{14} (*C*), or anti-acetyl-Lys^9 (*D*) as indicated. c-*fos* promoter DNA sequences were detected by quantitative real time PCR. The data are presented as percentages of total input. *E*, acid-soluble proteins were extracted from quiescent IKK wild-type (*WT*), IKK$\alpha^{-/-}$, and IKK$\alpha^{+/+}$ MEFs and immunoblotted with anti-phospho-Ser^{10}, anti-acetyl-Lys^9, or anti-acetyl-Lys^{14} H3 antibodies. Coomassie staining of acid-extracted histones was used to visualize equal loading.

gene expression in $p65^{-/-}$ MEFs following EGF treatment. $p65^{-/-}$ MEFs exhibited a marked defect in c-*fos* gene induction after EGF treatment in contrast to wild-type MEFs that show rapid induction of c-*fos* mRNA within 15 min of EGF stimulation (Fig. 5*B*). These results indicate that p65 may play a direct regulatory role in controlling c-*fos* gene expression or may facilitate promoter-associated IKKα or other factors to stimulate gene expression.

Association of IKKα at the c-fos Promoter Occurs Independently of the p65 NF-κB Subunit—The data described above raise the question of whether p65 is required for the association of IKKα with the c-*fos* promoter. To address this point, ChIP assays were performed in $p65^{-/-}$ MEFs. Quiescent $p65^{-/-}$ MEFs were left untreated or were treated with EGF along a time course followed by ChIP analysis with antibodies against IKKα or phospho-Ser^{10} histone H3. Our data demonstrate inducible levels of H3 Ser^{10} phosphorylation in response to EGF, with peak levels after 15 min of EGF treatment (Fig. 5*C*). Interestingly, peak levels of promoter-associated IKKα were detected after 10 min of EGF treatment, indicating that recruitment of IKKα at the c-*fos* promoter is p65-independent (Fig. 5*C*). The results were confirmed by quantification of ChIP DNA by real time PCR to measure the amount of c-*fos* genomic DNA that co-immunoprecipitated with the H3 Ser^{10} phosphorylation levels in $p65^{-/-}$ MEFs as well as wild-type MEFs relative to total input chromatin (data not shown). Collectively,

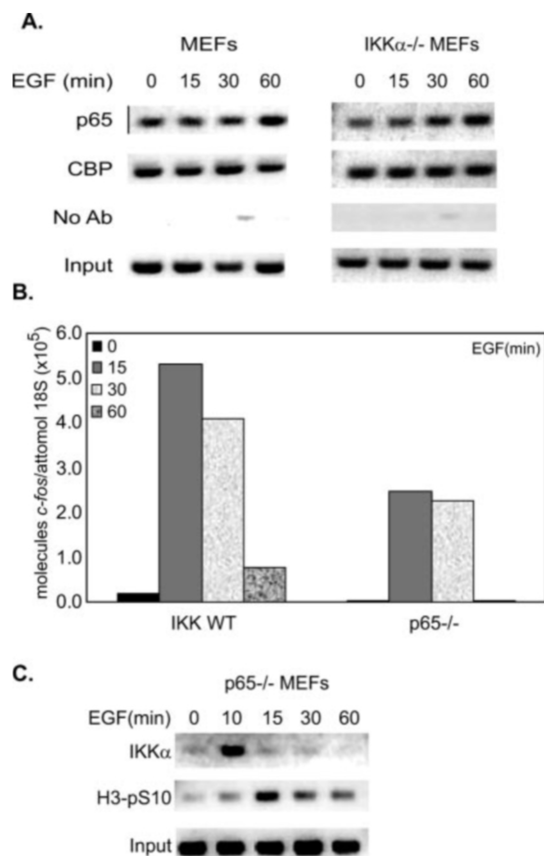

FIG. 5. **Promoter-associated p65/RelA contributes to the induction of c-*fos* gene expression.** *A*, chromatin was prepared from quiescent IKK wild-type (*left panel*) or IKK$\alpha^{-/-}$ (*right panel*) MEFs that were treated with EGF (50 ng/ml) at the indicated time points. ChIP assays were performed using anti-p65 or anti-CBP as indicated. c-*fos* promoter DNA sequences were detected by semi-quantitative PCR. *B*, real time PCR analysis was performed to measure the endogenous levels of c-*fos* mRNA in EGF stimulated wild-type or p65$^{-/-}$ MEFs. Quiescent cells were treated with EGF at the indicated time points, similar to *A*. The values are reported as molecules of c-*fos* per attomol of 18 S rRNA copies. *C*, EGF-induced recruitment of IKKα at the c-*fos* promoter occurs independently of p65. Chromatin was prepared from quiescent p65$^{-/-}$ MEFs that were treated with EGF (50 ng/ml) at the indicated time points. ChIP assays were performed using anti- IKKα or anti-phospho-Ser^{10} as indicated. c-*fos* promoter DNA sequences were detected by semi-quantitative PCR.

these results indicate that EGF-induced recruitment of IKKα to the c-*fos* promoter occurs independently of p65/RelA.

DISCUSSION

The results presented in this report indicate that IKKα is a critical regulator of mitogenic-induced H3 Ser^{10} phosphorylation, at least for the c-*fos* promoter. The data also support previously published work regarding the association between inducible phosphorylation and acetylation at certain promoters (17), whereby the loss of H3 Ser^{10} phosphorylation reduces but does not eliminate inducible levels of acetylation of histone H3 in IKK$\alpha^{-/-}$ MEFs. However, we do not exclude the possibility that these dynamic modifications occur independently as previously reported for the c-*jun* promoter (24). In addition to these dynamic modifications, interplay among other modifications, such as histone H3 methylation, may differentially contribute to c-*fos* gene regulation either positively or negatively (25, 26). The experiments also provide an interesting regulatory model whereby NF-κB controls gene expression in a manner that is independent of inducible promoter association of the NF-κB subunit, p65/RelA.

Evidence that IKKα controls c-*fos* histone H3 phosphorylation and inducible gene expression led us to address the potential association of the p65 NF-κB subunit with the c-*fos* promoter, because it was previously found that IKKα association with certain cytokine-regulated promoters was dependent on the recruitment of p65 (20, 21). Our current studies indicate that p65 is found constitutively associated with c-*fos* promoter. In this regard, the association of p65 with the c-*fos* promoter occurs in a manner that is similar to other c-*fos* regulatory factors (such as SRF) and that is different from the traditional mechanism of NF-κB regulation. Although others have observed the classical NF-κB activation response in cells that express high levels of EGF receptor (8), at least in quiescent MEFs, we did not observe any significant degradation of IκBα or nuclear accumulation of p65 following treatment with EGF. Interestingly, p65 contributed to the induced levels of c-*fos* gene expression following EGF treatment of quiescent MEFs (Fig. 5*B*). Thus, the role of the p65 subunit in controlling EGF-induced gene expression is not dependent on the enhanced recruitment of this transcription factor with its gene target. However, the mode of p65 activation downstream of EGF and the role that p65 plays in regulating c-*fos* gene expression are presently unclear.

The basis for the association of p65 with the c-*fos* promoter is unknown. Previous studies have suggested that p65 might participate in regulating the transcriptional activity of SRF, and a physical interaction was reported between p65 and SRF *in vitro* (27). Thus, it is possible that p65 is constitutively associated with the c-*fos* promoter through interactions with SRF. Additionally, we identified a previously uncharacterized NF-κB consensus site in the c-*fos* promoter (positions −214 to −223). The position of the region amplified with c-*fos* promoter-specific primer pairs used in the PCR step of the ChIP assays includes this putative NF-κB site. Electrophoretic mobility shift assays did not detect NF-κB DNA binding to this site in the c-*fos* promoter (data not shown), suggesting that this site is not a high affinity site for p65 *in vitro* and supports the possibility that p65 may function in concert with other nuclear factors at this promoter *in vivo*. Possibilities for promoter-associated p65 include cooperative interactions with SRF resulting in the formation of higher transcriptional complexes, as described above. In fact, antibodies to p65 (but not to p50) can partially supershift an SRF/SRE complex using a gel mobility shift assay (data not shown).

Although the importance of chromatin modification, including histone phosphorylation, in regulating gene expression is well recognized (28–30), the regulation of and identity of factors that control these modifications often remain unclear. Recent evidence, using MSK1/2 double knock-out MEFs, indicate that MSK proteins control global levels of H3 Ser^{10} phosphorylation as well as inducible H3 Ser^{10} phosphorylation at the c-*jun* promoter in response to mitogenic or stress stimuli (19). Furthermore, our studies and those of Soloaga *et al.* (19) indicate that the loss of inducible H3 Ser^{10} phosphorylation results in deficient c-*fos* gene expression levels. It is intriguing to speculate that this reduction may reflect the contribution of H3 Ser^{10} phosphorylation to immediate early gene expression. However, MSK1/2 and IKKα may play other roles in controlling c-*fos* gene expression. For example, MSK1 may play a role in activating the function of p65 at the c-*fos* promoter, because it has been proposed that MSK1 controls the phosphorylation of p65 on Ser^{276} (31), an event important in the interaction of p65 with co-activators, such as CBP (32). This concept could reflect a role for p65 and for MSK at the c-*fos* promoter in controlling expression of immediate-early genes. Longer term experimentation is directed toward understanding the complex regulatory network involving MSK1/2 and IKKα in controlling chromatin

modification and ultimately inducible gene expression.

The loss of p65 did not affect promoter-associated IKKα or EGF-induced H3 Ser^{10} phosphorylation at the c-*fos* promoter (Fig. 5*C*). This result was surprising as compared with our previous findings and those by Yamamoto *et al.* (21) where recruitment of IKKα to cytokine-inducible promoters is required the presence of p65. Thus, the mechanism of IKKα recruitment to the c-*fos* promoter could be dependent on the presence of another NF-κB subunit or via interactions with a distinct factor. It is important to note that IKKα interacts with the transcriptional co-activator CBP (21) and that this interaction may underlie promoter recruitment. In this regard, MAPK-directed phosphorylation of Elk-1 that stabilizes CBP/p300 interactions with the SRE ternary complex (14) may also lead to enhanced association of IKKα with the c-*fos* promoter.

Collectively, our findings provide the first evidence that promoter association of IKKα can occur in a p65-independent manner and that both IKKα and p65 contribute to EGF-induced c-*fos* gene expression. In this regard, IKKα has been shown to control keratinocyte differentiation in a manner that is independent of NF-κB (33). Additionally, IKKα knock-out mice exhibited skin and skeletal abnormalities (34), consistent with the effect on keratinocyte differentiation. Overall, our results indicate that both IKKα and p65 contribute toward maximal EGF-induced c-*fos* gene expression, potentially through distinct mechanisms. This is consistent with previous reports highlighting the complexity of c-*fos* gene regulation through multiple promoter-bound factors and/or specific signaling pathways (10).

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REFERENCES

1. Ghosh, S., and Karin, M. (2002) *Cell* **109,** S81–S96
2. Silverman, N., and Maniatis, T. (2001) *Genes Dev.* **15,** 2321–2342
3. Li, Q., and Verma, I. M. (2002) *Nat. Rev. Immunol.* **2,** 725–734
4. Yamamoto, Y., and Gaynor, R. (2002) *Curr. Mol. Med.* **1,** 287–296
5. Baldwin, A. S. (2001) *J. Clin. Invest.* **107,** 241–246
6. Pawson, T. (1995) *Nature* **373,** 573–580
7. Hill, C. S., and Treisman, R. (1995) *Cell* **80,** 199–211
8. Biswas, D., Cruz, A., Gansberger, E., and Pardee, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8542–8547
9. Clayton, A., and Mahadevan, L. (2003) *FEBS Lett.* **546,** 51–58
10. Treisman, R. (1995) *EMBO J.* **14,** 4905–4913
11. Janknecht, R., Ernst, W., Pingoud, V., and Nordheim, A. (1993) *EMBO J.* **12,** 5097–5104
12. Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M., and Shaw, P. (1995) *EMBO J.* **14,** 951–962
13. Janknecht, R., and Nordheim, A. (1996) *Biochem. Biophys. Res. Commun.* **228,** 831–837
14. Li, Q., Yang, S., Maeda, Y., Sladek, F., Sharrocks, A., and Martins-Green, M. (2003) *EMBO J.* **22,** 281–291
15. Chadee, N., Hendzel, M., Tylipski, C., Allis, C. D., Bazett-Jones, D., Wright, J., and Davie, J. (1999) *J. Biol. Chem.* **274,** 24914–24920
16. Clayton, A., Rose, S., Barratt, M., and Mahadevan, L. (2000) *EMBO J.* **19,** 3714–3726
17. Cheung, P., Tanner, K., Cheung, W., Sasson-Corsi, P., Denu, J., and Allis, C. D. (2000) *Mol. Cell* **5,** 905–915
18. Sassone-Corsi, P., Mizzen, C. A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C. D. (1999) *Science* **285,** 886–891
19. Soloaga, A., Thomson, S., Wiggen, G., Rampersaud, N., Dyson, M., Hazzalin, C., Mahadevan, L., and Arthur, J. (2003) *EMBO J.* **22,** 2788–2797
20. Anest, V., Hanson, J., Cogswell, P., Steinbrecher, K., Strahl, B., and Baldwin, A. (2003) *Nature* **423,** 659–663
21. Yamamoto, Y., Verma, U., Prajapati, S., Kwak, Y.-T., and Gaynor, R. (2003) *Nature* **423,** 655–659
22. Li, X., Massa, P. E., Hanidu, A., Peet, G. W., Aro, P., Savitt, A., Mische, S., Li, J., and Marcu, K. B. (2002) *J. Biol. Chem.* **277,** 45129–45140
23. Murai, K., and Treisman, R. (2002) *Mol. Cell. Biol.* **22,** 7083–7092
24. Thomson, S., Clayton, A. L., and Mahadevan, L. C. (2001) *Mol. Cell.* **6,** 1231–1241
25. Zhang, Y., and Reinberg, D. (2001) *Genes Dev.* **15,** 2343–2360
26. Fischle, W., Wang, Y., and Allis, C. D. (2003) *Nature* **425,** 475–479
27. Franzoso, G., Carlson, L., Brown, K., Daucher, M., Bressler, P., and Siebenlist, U. (1996) *EMBO J.* **15,** 3403–3412
28. Strahl, B., and Allis, C. D. (2000) *Nature* **401,** 41–45
29. Berger, S. (2001) *Oncogene* **20,** 3007–3013
30. Narlikar, G., Fan, H., and Kingston, R. E. (2002) *Cell* **108,** 475–487
31. Vermeulen, L., De Wilde, G., Van Damme, P., Vandern Berghe, W., and Haegeman, G. (2003) *EMBO J.* **22,** 1313–1324
32. Zhong, H., May, M., Jimi, E., and Ghosh, S. (2002) *Mol. Cell* **9,** 625–636
33. Hu, Y., Baud, V., Oga, T., Kim, K., Yoshida, Y., and Karin, M. (2001) *Nature* **410,** 710–714
34. Li, Q., Lu, Q., Hwang, S., Buscher, D., Lee, K., Izpisua-Belmonte, J., and Verma, I. (1999) *Genes Dev.* **13,** 1322–1328