Histone H3 phosphorylation by IKK-α is critical for cytokine-induced gene expression

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Cytokine-induced activation of the IκB kinases (IKK) IKK-α and IKK-β is a key step involved in the activation of the NF-κB pathway[1–4]. Gene-disruption studies of the murine IKK genes have shown that IKK-β, but not IKK-α, is critical for cytokine-induced IκB degradation[5–7]. Nevertheless, mouse embryo fibroblasts deficient in IKK-α are defective in the induction of NF-κB-dependent transcription[7–9]. These observations raised the question of whether IKK-α might regulate a previously undescribed step to activate the NF-κB pathway that is independent of its previously described cytoplasmic role in the phosphorylation of IκBα. Here we show that IKK-α functions in the nucleus to activate the expression of NF-κB-responsive genes after stimulation with cytokines. IKK-α interacts with CREB-binding protein and in conjunction with Rel A is recruited to NF-κB-responsive promoters and mediates the cytokine-induced phosphorylation and subsequent acetylation of specific residues in histone H3. These results define a new nuclear role of IKK-α in modifying histone function that is critical for the activation of NF-κB-directed gene expression.

First, we characterized cytokine-induced activation of the NF-κB pathway in parental, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ mouse embryo fibroblasts (MEFs). Tumour-necrosis factor-α (TNF-α) induced the rapid degradation of IκBα in both parental MEF cells and in IKK-$\alpha^{-/-}$ cells but not in IKK-$\beta^{-/-}$ cells (Fig. 1a). However, defects in the expression of an NF-κB reporter were noted after treatment with TNF-α in both IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells without changes in the expression of a Rous sarcoma virus–β-galactosidase reporter (Fig. 1b). Furthermore, quantitative real-time polymerase chain reaction (PCR) analysis confirmed that TNF-α increased the transcription of the NF-κB-regulated IκBα gene in both MEF and IKK-$\alpha^{+/+}$ cells, in which Myc-tagged IKK-α was stably expressed in IKK-$\alpha^{-/-}$ cells but not in IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells (Fig. 1c). These results indicated that IKK-α is critical for the rapid cytokine-mediated induction of NF-κB-responsive genes by a mechanism distinct from that of IKK-β-mediated increases in IκBα degradation.

It has recently been noted that IKK-α can shuttle between cytoplasm and nucleus[10]. To address whether the intracellular distribution of IKK-α and IKK-β was different, these endogenous proteins were immunostained in HeLa cells (Fig. 1d) in addition to both parental and IKK-deficient mouse embryo fibroblasts (Fig. 1e). IKK-α had a different distribution from that of IKK-β: IKK-α was localized in both the cytoplasm and the nucleus, whereas IKK-β was localized predominantly in the cytoplasm (Fig. 1d, e). These results indicate that IKK-α might have an additional nuclear role in activating the NF-κB pathway that is distinct from its previously described role in phosphorylating the IκB proteins.

To investigate whether IKK-α might function in the nucleus to regulate the cytokine-induced expression of the NF-κB-responsive IκBα and interleukin-8 (IL-8) genes, chromatin immunoprecipitation (ChIP) assays were used. HeLa cells were treated with TNF-α and ChIP assays were performed at various times after stimulation. In response to stimulation with TNF-α, the recruitment of IKK-α, p65 and CREB binding protein (CBP) to the IκBα and IL-8 promoters was detected 15 minutes after treatment and was present for at least 120 minutes (Fig. 2a, left and middle panels). In contrast, there was no association of either IKK-β or IKK-γ/NEMO with these promoters unless several additional PCR cycles were used (Fig. 2a, left and middle panels). There was no cytokine-induced association of any of these factors with the β-actin promoter (Fig. 2a, right panel). Quantitative real-time PCR analysis was used to determine the percentage of IκBα and IL-8 promoter input DNA that was bound by IKK-α, p65 and CBP (Fig. 2b). Finally, the kinetics of binding of these factors to the IκBα and IL-8 genes was correlated with their increased transcription (Fig. 2c).

Given the fact that interactions between CBP and p65 have been shown to be important for NF-κB activation[11–14], it was possible that IKK-α might form a complex with these proteins on NF-κB-regulated promoters to increase transcription. IKK-α might then activate NF-κB-regulated transcription by the phosphorylation of p65 (refs 15, 16) or potentially other nuclear targets. First, the interactions of epitope-tagged proteins including CBP and either IKK-α, IKK-β or p65 were analysed in extracts prepared from cells co-transfected with expression vectors encoding these proteins. Immunoprecipitation and subsequent western blot analysis

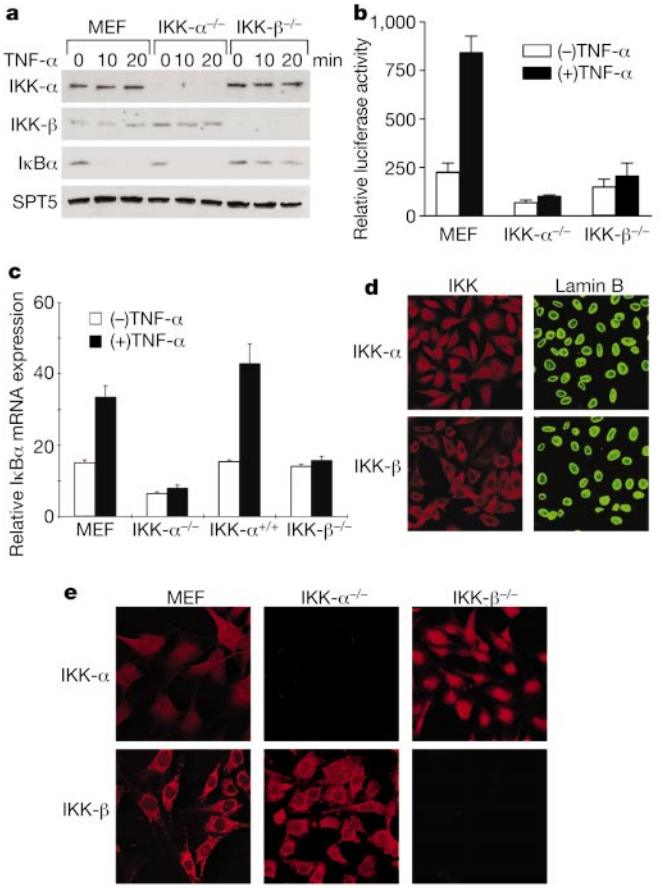

Figure 1 Both IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells are defective in TNF-α-induced NF-κB activation. **a**, Cells were treated with TNF-α for the specified durations, and western blot analysis of these extracts was performed with the indicated antibodies. **b**, TNF-α-induced NF-κB activation was determined by luciferase assays after transfection of an NF-κB-dependent reporter construct and normalized by a Rous sarcoma virus–β-galactosidase reporter construct. **c**, The indicated cell lines were either untreated or treated with TNF-α for 15 min and the change in IκBα mRNA expression was determined by quantitative real-time PCR and normalized by measuring the levels of 18S RNA. **d**, Immunofluorescence of HeLa cells and **e**, of MEF, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells was performed with the indicated IKK-α-specific and IKK-β-specific antibodies.

demonstrated that there were strong interactions between IKK-α and CBP as well as between p65 and CBP (Fig. 3a). In addition, the mammalian two-hybrid assay demonstrated that IKK-α strongly interacted with the amino-terminal transactivation domain of CBP, which is also the binding site for p65 (Fig. 3b)[17]. However, no direct interaction was detected between IKK-α and p65 in this analysis (data not shown). Consistent with the two-hybrid data is the observation that IKK-α bound to a glutathione *S*-transferase (GST)–CBP fusion protein containing the N terminus of CBP (Fig. 3c). Similar data demonstrating the interaction of IKK-α and p65, but not IKK-β, with CBP were also seen with HeLa extracts containing either these transiently expressed proteins (Fig. 3d), or endogenous proteins (Fig. 3e).

It was important to determine whether p65 was required for the association of IKK-α with the IκBα promoter. For these studies we used infection with adenoviruses encoding either the IκB super-repressor[18,19], which expresses an IκB protein that is not degraded after stimulation by cytokine and prevents both p65 nuclear translocation and DNA binding, or β-galactosidase as a control (Fig. 4a). ChIP assays demonstrated that the IκBα super-repressor prevented the association of p65, but not IKK-α or CBP, with the IκBα promoter (Fig. 4a). Although these results suggested that p65 was not crucial for IKK-α association, we found defects in the association of both IKK-α and CBP, in addition to other factors that bound to the IκBα and IL-8 promoter, in ChIP assays performed in TNF-α-treated $p65^{-/-}$ cells (data not shown). It remains to be determined whether the defect in IKK-α and CBP promoter recruitment seen in $p65^{-/-}$ cells was due only to the failure of p65 to bind to NF-κB-regulated promoters or to other effects.

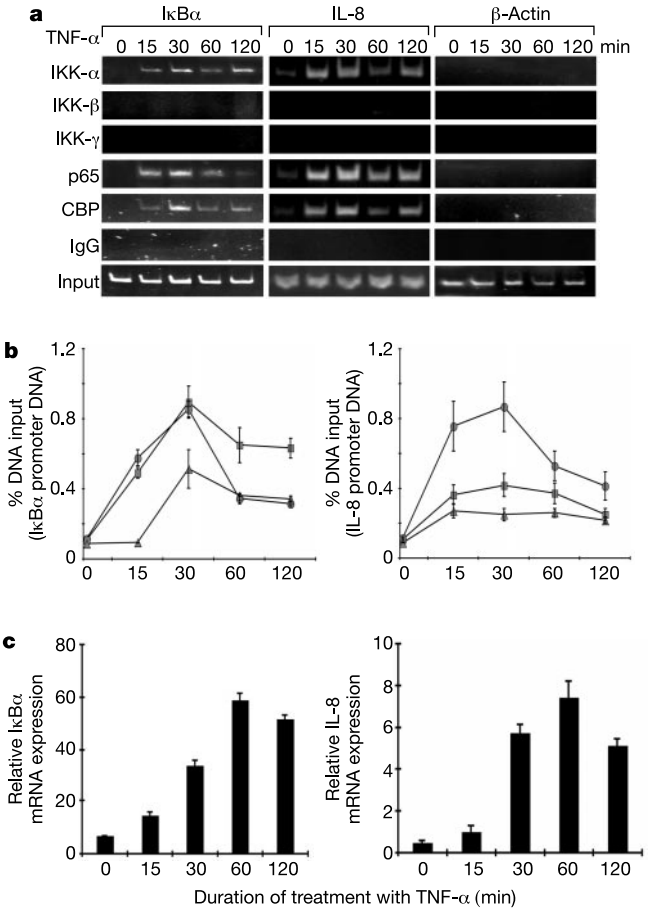

Figure 2 TNF-α treatment leads to IKK-α association with the IκBα and IL-8 promoters. **a**, HeLa cells were treated with TNF-α, and chromatin immunoprecipitation assays were performed with the indicated antibodies. The detection of the immunoprecipitated IκB promoter (left panel), IL-8 promoter (middle panel) or β-actin promoter (right panel) was analysed by PCR with promoter-specific primers. **b**, Quantitative real-time PCR was performed to determine the kinetics of factor binding to these promoters in comparison with input DNAs. Squares, IKK-α; circles, p65; triangles, CBP. **c**, IκBα and IL-8 mRNA levels were determined by quantitative real-time PCR and normalized by measuring the levels of 18S RNA.

Next we addressed whether intact IKK-α kinase activity was required for association with the IκBα promoter. Both the epitope-tagged wild-type and mutant IKK-α proteins were recruited to the IκBα promoter after treatment with TNF-α (Fig. 4b). The ability of these IKK-α proteins to alter CBP-mediated gene expression directly was next addressed. Transfection of wild-type IKK-α, but not the kinase-dead IKK-α(K/M) mutant or IKK-β, dose-dependently enhanced GAL4–CBP-mediated transcriptional activity (Fig. 4c). These results suggested that although both wild-type and mutant IKK-α proteins could associate with the IκBα promoter, IKK-α kinase activity was important for modulating CBP-dependent transcription.

These observations raised the question of the mechanism by which IKK-α could modulate CBP-dependent transcriptional activation. The histone acetyltransferase (HAT) activity of CBP acetylates the N-terminal tails of histones such as H3 during transcriptional activation[20–23]. One potential effect of histone H3 phosphorylation has been proposed to be the recruitment and activation of HAT[22,23]. For example, the phosphorylation of Ser 10 in histone H3 enhances the HAT-mediated acetylation of Lys 14. Such modifications of specific residues in the N-terminal tails of histones might serve as a signal for the binding of specific co-activators to result in increased gene expression.

Because the kinase that regulates the cytokine-induced phosphorylation of histone H3 has not been identified, we addressed whether IKK-α might be involved in this process. The state of acetylation and phosphorylation of histone H3 in MEF, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells that had been serum-starved for 16 h before treatment with TNF-α was characterized. Treatment of MEF cells with TNF-α resulted in an increased phosphorylation of Ser 10 and acetylation of Lys 14 in histone H3 (Fig. 5a). In IKK-$\alpha^{-/-}$ cells there was a significantly decreased phosphorylation of Ser 10 and acetylation of Lys 14 in histone H3 both in the absence and in the presence of TNF-α in comparison with that seen in MEF cells (Fig. 5a). IKK-$\beta^{-/-}$ cells contained similar levels of histone H3 phosphorylation to that detected in MEF cells, but exhibited marked decreases in the acetylation of Lys 14 in histone H3. Treatment of each of these cells with trichostatin A, an inhibitor of histone deacetylases, resulted in similar levels of histone acetylation. These results suggested that IKK-α may play a role in phosphorylation of Ser 10, which might be important for the subsequent acetylation of Lys 14 in histone H3.

Next, kinase assays were performed *in vitro* to determine whether IKK-α could directly phosphorylate histone H3. Kinase assays performed with purified histone H3 as substrate demonstrated that wild-type IKK-α, but not the kinase-defective mutants IKK-α(SS/AA) or IKK-α(K/M), resulted in enhanced histone H3 phosphorylation (Fig. 5b). Consistent with these results was the observation from western blot analysis that the presence of IKK-α resulted in increased levels of phosphorylated Ser 10 and acetylated Lys 14 in histone H3 (Fig. 5c). ChIP assays demonstrated that IKK-α led to the increased association with the IκBα promoter of histone H3 that was phosphorylated on Ser 10 and acetylated on Lys 14 without changing the association of total histone H3 and phosphorylated histone H1 (Fig. 5d). These results indicated that IKK-α was likely to be responsible for cytokine-induced phosphorylation and subsequent acetylation in histone H3.

Finally, we addressed the kinetics of TNF-α-mediated increases in the association of IKK-α, p65, CBP and phosphorylated and acetylated histone H3 with the IκBα promoter (Fig. 5e). ChIP

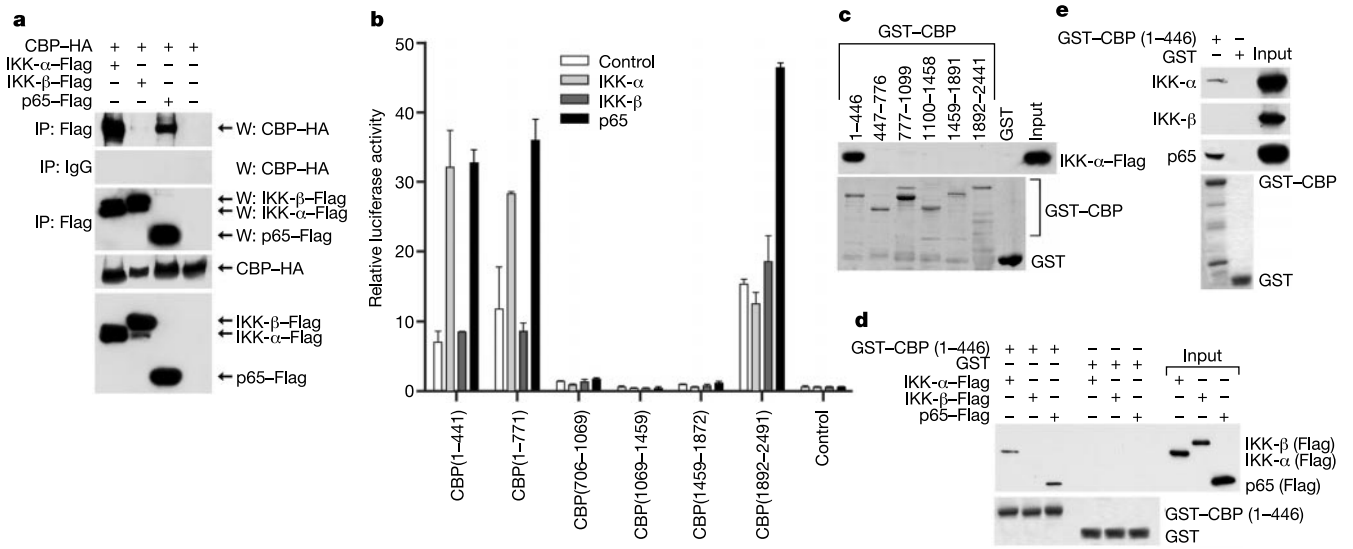

Figure 3 IKK-α interacts with the CBP transactivation domain. **a**, Flag-tagged IKK-α, IKK-β or p65 was co-transfected with haemagglutinin (HA)-tagged CBP into HEK-293 T cells. After immunoprecipitation (IP) with anti-Flag antibody, the immunoprecipitates were analysed by immunoblotting (W) with anti-HA or anti-Flag antibody as indicated. **b**, The indicated regions of CBP fused to the GAL4 DNA-binding domain were co-transfected with a GAL4 luciferase reporter construct and IKK-α, IKK-β or p65 constructs containing the VP16 activation domain. **c**–**e**, GST–CBP fusion proteins were used to determine their interaction with Flag-tagged IKK-α, IKK-β or p65 in extracts prepared from transfected cells (**c** and **d**) or with endogenous proteins isolated from HeLa cells (**e**).

assays of MEF and IKK-$\alpha^{-/-}$ cells demonstrated that TNF-α treatment led to the recruitment of both p65 and CBP to the IκBα promoter (Fig. 5e). Whereas treatment of MEF cells with TNF-α led to the enhanced promoter association of histone H3 phosphorylated on Ser 10 and acetylated on Lys 14, little or no promoter association of histone H3 modified in this manner was found in IKK-$\alpha^{-/-}$ cells. ChIP assays of the IκBα promoter in IKK-$\beta^{-/-}$ cells required the addition of threefold more input DNA to facilitate the detection of factor binding than in the other cell lines, owing to the decreased association of IKK-α, CBP and p65 with the IκBα promoter (Fig. 5e). Treatment of these cells with TNF-α resulted in an increased association of histone H3

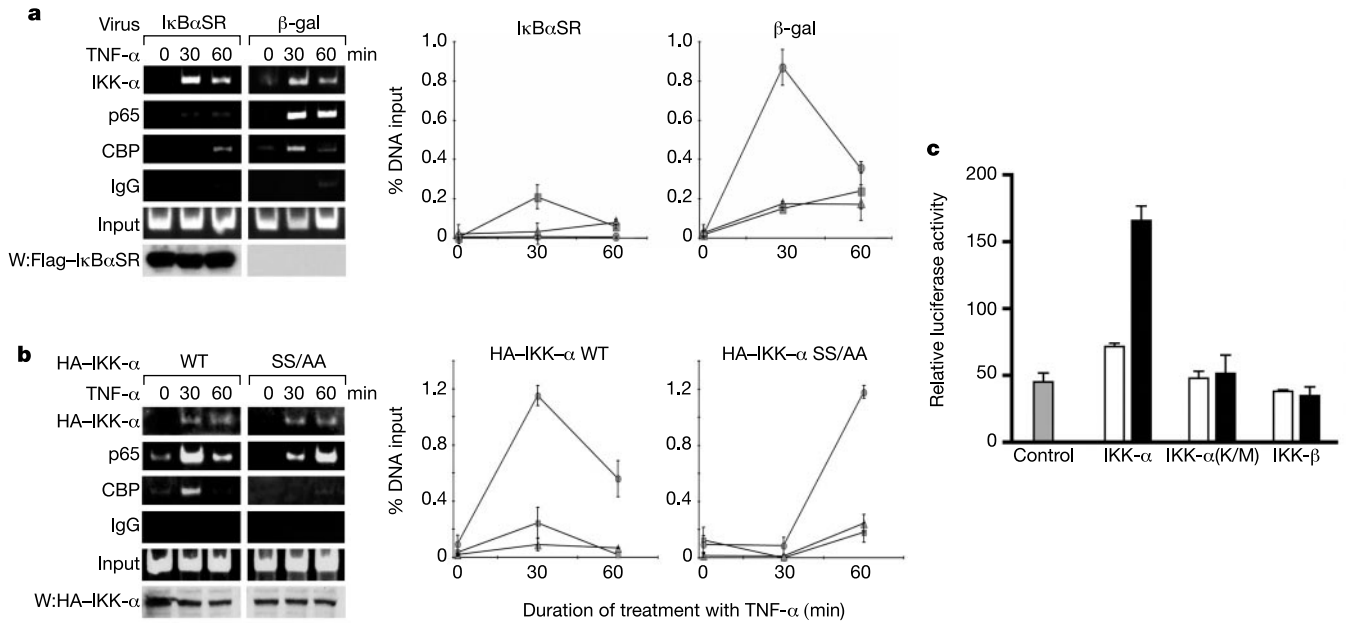

Figure 4 IKK-α promoter association is not strictly dependent on p65 binding and results in increased CBP transcriptional activity. **a**, HeLa cells infected with adenovirus encoding either the IκBα super-repressor (IκBαSR) or β-galactosidase (β-gal) were stimulated with TNF-α for the specified durations. The association of IKK-α, p65 and CBP with the IκBα promoter was analysed by ChIP assays followed by PCR analysis (left panel) or quantitative real-time PCR (right panels). Squares, IKK-α; circles, p65; triangles, CBP. **b**, After transfection of either haemagglutinin (HA)-tagged IKK-α (WT) or IKK-α(SS/AA) into HeLa cells, ChIP assays (left panel) and quantitative real-time PCR (right panels) were performed. Squares, IKK-α; circles, p65; triangles, CBP. Immunoblotting (W) of the epitope-tagged proteins is also indicated. **c**, An expression vector encoding a GAL4–CBP fusion protein was transfected with a GAL4 luciferase reporter construct and either 0.1 μg (open bars) or 0.5 μg (filled bars) of expression vectors encoding IKK-α, IKK-α(K/M) or IKK-β tagged with Myc, and CBP-dependent transcriptional activity was determined by luciferase assays.

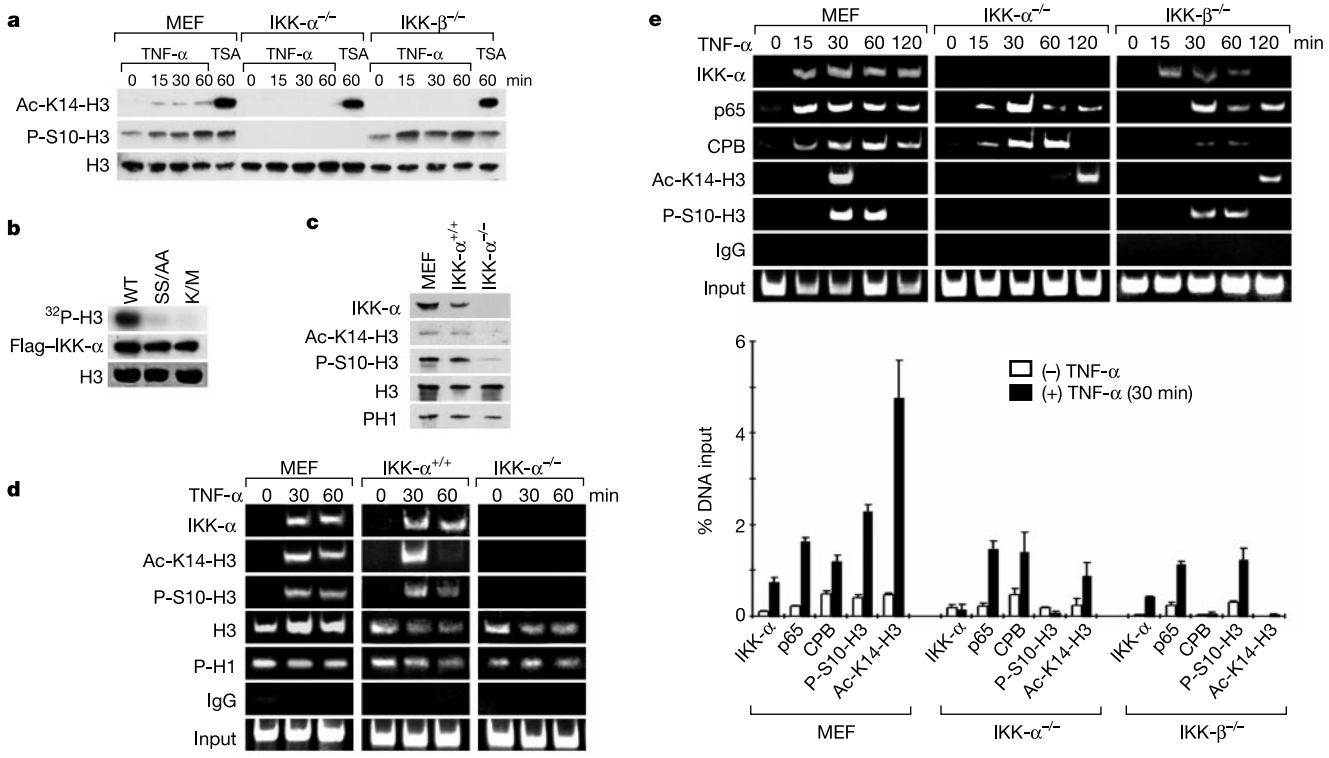

Figure 5 Requirement for IKK-α in histone H3 phosphorylation. **a**, Each of the indicated cell lines was serum-starved for 16 h, then treated with TNF-α for the specified durations or with trichostatin A (TSA) for 60 min. Acid-extracted proteins from these cells were immunoblotted with the indicated antibodies. **b**, Flag-tagged IKK-α, IKK-α(SS/AA) or IKK-α(K/M) constructs were transfected into HeLa cells and the kinase activity of these immunoprecipitated Flag-tagged proteins was determined with purified histone H3 as substrate. **c**, Extracts prepared from IKK-$\alpha^{-/-}$, IKK-$\alpha^{+/+}$ or MEF cells were analysed for IKK-α expression and the acid-extracted proteins from these cells were immunoblotted with the indicated antibodies. **d**, The association of these factors with the IκBα promoter was also determined by ChIP assays (lower panel). **e**, MEF, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ were treated with TNF-α for the specified durations and ChIP assays were performed and analysed by PCR (top panel) or quantitative real-time PCR at 0 and 30 min after treatment with TNF-α (lower panel).

phosphorylated on Ser 10, but a markedly reduced association of both CBP and histone H3 acetylated on Lys 14 compared with that seen in MEF cells (Fig. 5e). These studies suggested that the binding of both IKK-α and CBP to the IκBα promoter was important for the association of histone H3 phosphorylated on Ser 10 and acetylated on Lys 14.

Phosphorylation of histone H3 is associated with several distinct biological processes including the mitogen-induced and cytokine-induced activation of specific genes and the compaction of chromosomes at the onset of mitosis[20,21]. For example, the Aurora kinases have been shown to be important in the phosphorylation of Ser 10 in histone H3 during mitosis[21]. Stimulation of both the extracellular signal-regulated protein kinase (ERK) pathway and the stress-activated p38 pathway also result in an increase in phosphorylation of histone H3 (Ser 10) during the activation of immediate-early gene expression[20,21]. The Rsk-2 (ref. 24) and Msk-1 (ref. 25) kinases have been shown to be important in this process. Previous studies of the IκBα promoter indicated that an uncharacterized kinase distinct from Rsk-2 and Msk-1 could phosphorylate histone H3 during cytokine-mediated activation of this promoter[26]. Our analysis and that of a companion paper[27] indicate that IKK-α, but not IKK-β, is likely to be the kinase that is critical in facilitating the rapid cytokine-induced expression of NF-κB-regulated genes.

IKK-α recruitment to NF-κB regulated promoters is probably mediated by multiple promoter-bound factors including components of the basal transcription complex, co-activators such as CBP, and perhaps chromatin itself. The cytokine-induced phosphorylation by IKK-α of Ser 10 in histone H3 seems to be especially important for the subsequent acetylation of Lys 14 by CBP. This result is consistent with previous studies indicating that phosphorylation of Ser 10 precedes the subsequent acetylation of Lys 14 in histone H3 and that the HAT activity for H3 phosphorylated on Ser 10 is enhanced in comparison with that for unmodified H3 (refs 22, 23). In addition to its previously described role on the processing of p100 to p52 (ref. 28), our analysis shows that IKK-α is important in regulating a critical step required for NF-κB-dependent gene expression. The studies presented here extend our knowledge of the multiple functions of the IκB kinases in activating the NF-κB pathway. □

Methods

Cells and reagents

Mouse embryo fibroblasts (MEFs) were a gift from Xiaodong Wong. IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells were provided by Inder M. Verma. A Moloney-based retrovirus expressing (MEQKLISEEDLN) (Myc)-tagged IKK-α was used with selection by hygromycin to stably express IKK-α in IKK-$\alpha^{-/-}$ cells to generate IKK-$\alpha^{+/+}$ cells. Antibodies directed against IKK-α/β, IκBα, p65 and CBP were obtained from Santa Cruz Biotechnology. IKK-α-specific antibody was from BD/Pharmingen and Oncogene and IKK-β-specific antibody was obtained from Cell Signal Technology. Antibodies directed against acetyl-histone H3(Lys 14), phospho-histone H3(Ser 10) and phospho-histone H1 were obtained from Upstate Biotechnology. Antibody against histone H3 was obtained from Cell Signal Technology. TNF-α (Roche Molecular Biochemicals) was used at a final concentration of 10 ng ml^{-1}, and trichostatin A (Sigma) was used at 500 ng ml^{-1}.

Quantitative real-time PCR

Total RNA was prepared from HeLa, MEF, IKK-$\alpha^{-/-}$, IKK-$\alpha^{+/+}$ or IKK-$\beta^{-/-}$ cells with the RNeasy kit (Qiagen) and treated with the DNA-free kit (Ambion) to remove residual genomic DNA. For real-time PCR, the cDNA was prepared with oligo(dT) and random primers (Invitrogen) and analysed in triplicate with the SYBR GreenMaster Mix (Applied Biosystems) for 15 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s in the ABI Sequence Detection System. The oligonucleotide

primers used to analyse IκBα transcripts (+2180 to +2378) contained the sequences 5′-GATCCGCCAGGTGAAGGG-3′ and 5′-GCAATTTCTGGCTGGTTGG-3′, the primers used to analyse IL-8 transcripts (+1 to +239) contained the sequences 5′-ATGACTTCCAAGCTGGCCGT-3′ and 5′-TTACATAATTTCTGTGTTGGC-3′, and the primers used to analyse the 18S ribosomal RNA contained the sequences 5′-AGGAATTGACGGAAGGGCAC-3′ and 5′-GGACATCTAAGGGCATCACA-3′.

ChIP assays

ChIP assays were performed with a previously described protocol (Upstate Biotechnology). In brief, chromatin from crosslinked cells was sheared by sonication (three times, 15 s each; one-third power) and incubated overnight with specific antibody followed by incubation with protein G–Sepharose saturated with salmon sperm DNA. Precipitated DNAs were analysed by quantitative PCR (34 cycles) with a *Taq* PCR Master mix kit (Qiagen) and primers for either the human 5′-GACGACCCCAATTCAAATCG-3′ and 5′-TCAGGCTCGGGGAATTTCC-3′ or murine 5′-GGACCCCAAACCAAAATCG-3′ and 5′-TCAGGCGCGGGGAATTTCC-3′ IκBα promoters (−316 to −15), together with the human IL-8 promoter (−121 to +61) 5′-GGGCCATCAGTTGCAAATC-3′ and 5′-TTCCTTCCGGTGGTTTCTTC-3′ and the human β-actin promoter (−980 to −915) 5′-TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGCCATAAAAGGCAA-3′. Quantitative real-time PCR was performed in triplicate to determine the association of IKK-α, p65 and CBP with the IκBα and IL-8 promoters by using 500 nM of the above oligonucleotide primers and input DNA standards diluted in threefold increments from 10% to 0.01% with SYBR Green Master Mix and the ABI Prism 7700 Sequence Detection System.

In vitro interaction assay

Fragments of the CBP coding sequence were cloned into pGEX vector (Pharmacia). Purified GST–CBP fusion proteins were immobilized to glutathione–agarose and incubated overnight with cell lysates (100 μg protein). After extensive washing with cold PBS, the protein complexes were analysed by immunoblotting.

IKK-α kinase assay

Total cell lysates (100 μg protein) prepared from cells transfected with expression vectors encoding Flag-tagged IKK-α, IKK-α(K/M) or IKK-α(SS/AA) were incubated for 1 h with anti-Flag antibody (Sigma) and with protein A–agarose for a further 1 h. After extensive washing of the immunoprecipitates, kinase assays were performed as described[29] with 5 μg of histone H3 (Sigma) as a substrate.

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A nucleosomal function for IκB kinase-α in NF-κB-dependent gene expression

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NF-κB is a principal transcriptional regulator of diverse cytokine-mediated processes and is tightly controlled by the IκB kinase complex (IKK-α/β/γ). IKK-β and IKK-γ are critical for cytokine-induced NF-κB function, whereas IKK-α is thought to be involved in other regulatory pathways[1–4]. However, recent data suggest a role for IKK-α in NF-κB-dependent gene expression in response to cytokine treatment[1,5–7]. Here we demonstrate nuclear accumulation of IKK-α after cytokine exposure, suggesting a nuclear function for this protein. Consistent with this, chromatin immunoprecipitation (ChIP) assays reveal that IKK-α was recruited to the promoter regions of NF-κB-regulated genes on stimulation with tumour-necrosis factor-α. Notably, NF-κB-regulated gene expression is suppressed by the loss of IKK-α and this correlates with a complete loss of gene-specific phosphorylation of histone H3 on serine 10, a modification previously associated with positive gene expression. Furthermore, we show that IKK-α can directly phosphorylate histone H3 *in vitro*, suggesting a new substrate for this kinase. We propose that IKK-α is an essential regulator of NF-κB-dependent gene expression through control of promoter-associated histone phosphorylation after cytokine exposure. These findings provide additional insight into the role of the IKK complex in NF-κB-regulated gene expression.

Characteristic cytokine-mediated activation of the NF-κB pathway involves IKK-β-directed phosphorylation of and subsequent degradation of inhibitors of NF-κB (IκBs), resulting in rapid nuclear accumulation of NF-κB subunits[1–3]. It has been found that IKK-α, but not IKK-β, constitutively shuttles between the cytoplasm and the nucleus, suggesting a nuclear function for this IKK subunit[8]. To address whether the subcellular localization of IKK-α changes on