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

cytokine exposure, nuclear and cytoplasmic extracts from mouse embryonic fibroblasts (MEFs) stimulated with tumour-necrosis factor-α (TNF-α) were immunoblotted with antibodies against p65 and the different IKK subunits. Notably, stimulation of MEFs with TNF-α results in marked nuclear accumulation of IKK-α (Fig. 1a). In contrast to IKK-α, both IKK-β and IKK-γ are present in the nucleus and the cytoplasm, and exhibit no appreciable changes on TNF-α induction (Fig. 1a). We demonstrated the purity of nuclear and cytoplasmic fractions with antibodies specific for p105, an NF-κB precursor known to be cytoplasmic, and for TFIIB, a known nuclear protein (Fig. 1a). Immunofluorescence staining with an anti-IKK-α antibody confirms the western analysis. IKK-α is predominantly cytoplasmic with low but detectable nuclear levels in unstimulated wild-type MEFs, whereas TNF-α-induced cells exhibit higher levels of nuclear IKK-α (Fig. 1b, left panel). Loss of immunoreactivity in IKK-$\alpha^{-/-}$ cells confirms antibody specificity (Supplementary Information). Consistent with previous reports, TNF-α treatment induces nuclear translocation of p65 (Fig. 1b, right panel). Although the kinetics of induced IKK-α nuclear accumulation are similar to those of p65, TNF-α induction leads to nuclear accumulation of IKK-α in p65$^{-/-}$ cells (data not shown), indicating that IKK-α does not require p65 for nuclear entry. These data suggest that IKK-α may have a nuclear function distinct from the canonical NF-κB activation pathway.

Consistent with previous reports[1], we observed normal TNF-α-induced IκBα degradation and induced p65 nuclear translocation in MEFs lacking IKK-α (data not shown). However, IκBα resynthesis is delayed in IKK-$\alpha^{-/-}$ cells compared with wild-type MEFs (Fig. 1c). The defect in resynthesis is consistent with a previous report implicating a role for IKK-α in TNF-α-induced IκBα gene expression[5]. Accordingly, we performed real-time polymerase chain reaction (PCR) analysis to examine the expression profile of the NF-κB-regulated IκBα gene (*Nfkbia*) in IKK-$\alpha^{-/-}$, IKK-$\beta^{-/-}$ and wild-type MEFs. TNF-α rapidly induces IκBα gene expression in wild-type MEFs (Fig. 2a), whereas TNF-α-induced IκBα gene expression is significantly decreased in both IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells. We next examined the kinetics of TNF-α-induced interleukin-6 (IL-6) gene expression by northern analysis. Both IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells are deficient in IL-6 gene (*Il6*) activation at all time points after TNF-α-induction (Fig. 2c). Previous reports have shown that loss of IKK-α and IKK-β results in altered transcriptional activation of IκBα, IL-6 and other NF-κB-dependent genes[5–7]. Thus, our results support a requirement for IKK-α and IKK-β in optimal IκBα and IL-6 gene expression.

The molecular mechanism(s) through which IKK-α regulates the expression of specific cytokine-induced NF-κB-dependent genes has not been determined previously. To investigate a nuclear role for IKK-α, we performed ChIP assays with antibodies directed against p65, IKK-α and IKK-β in TNF-α-stimulated wild-type MEFs. Furthermore, we used phosphorylated and acetylated histone H3 antibodies to examine histone H3 modifications that are known to correlate with active gene expression at the IκBα promoter[9]. The results reveal a rapid recruitment of p65 to the IκBα promoter in response to TNF-α (Fig. 2b, left panel). Of note, both IKK-α and IKK-β recruitment are observed after TNF-α-induction, whereas we detect a low level of IKK-α recruitment in unstimulated cells.

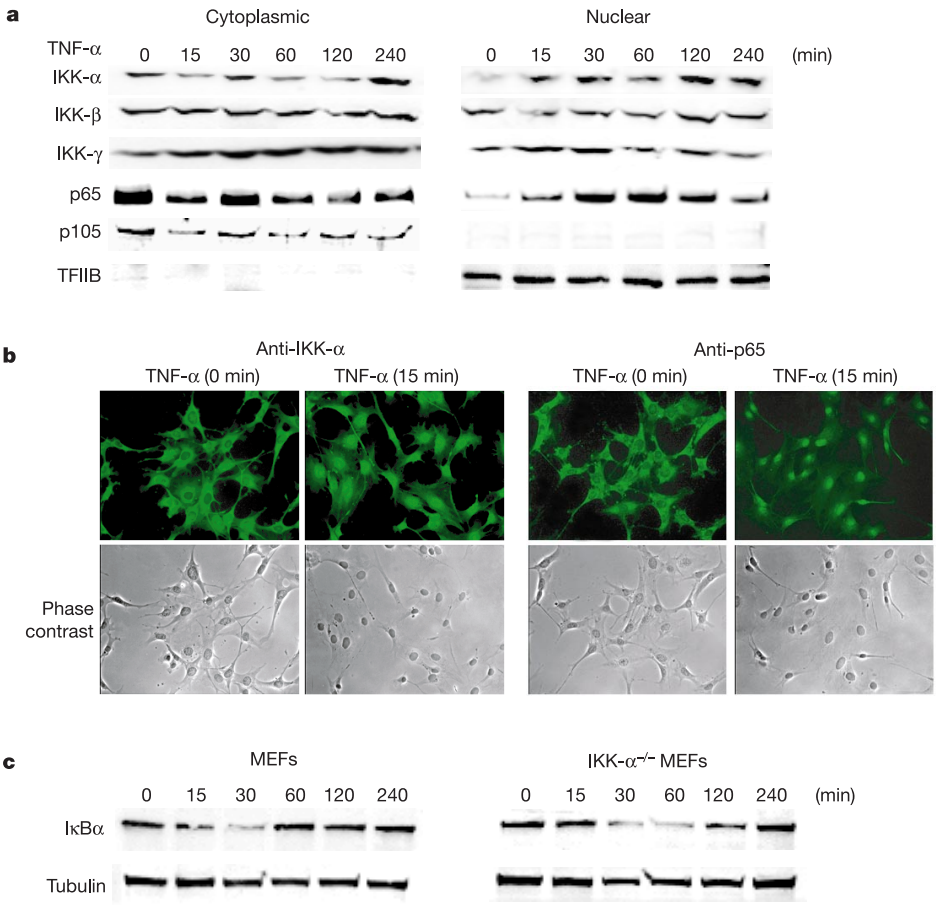

Figure 1 TNF-α-induced nuclear accumulation of endogenous IKK-α. **a**, MEFs were stimulated with TNF-α (10 ng ml^{-1}) and fractionated into nuclear and cytoplasmic fractions, and then analysed directly by western blotting with IKK-α, IKK-β, IKK-γ and p65 antibodies. Antibodies specific for cytoplasmic p105/p50 and nuclear TFIIB demonstrate the purity of these fractions. **b**, MEFs were treated for 15 min with TNF-α as indicated, and were processed for indirect immunofluorescence and stained with IKK-α or p65 antibodies. **c**, Kinetics of TNF-α-induced IκBα degradation in IKK wild-type and IKK-$\alpha^{-/-}$ MEFs. Cells were treated with TNF-α for the indicated times and western analysis was performed with anti-IκBα antibody.

The kinetics of IKK-α recruitment and histone H3 Ser 10 phosphorylation correlate in uninduced and TNF-α-induced cells. Consistent with previous reports and with evidence of basal IκBα gene expression[9–11], we observed constitutive levels of acetylated histone H3 at this promoter (Fig. 2b, left panel). Enrichment of promoter sequences is not detected at the β-actin promoter or when the antibody is omitted from the immunoprecipitation reaction, confirming the specificity of the ChIP assay (data not shown). These results demonstrate a TNF-α-inducible association of IKK-α and IKK-β with the IκBα promoter, and suggest a new function for these kinases in NF-κB-dependent gene regulation.

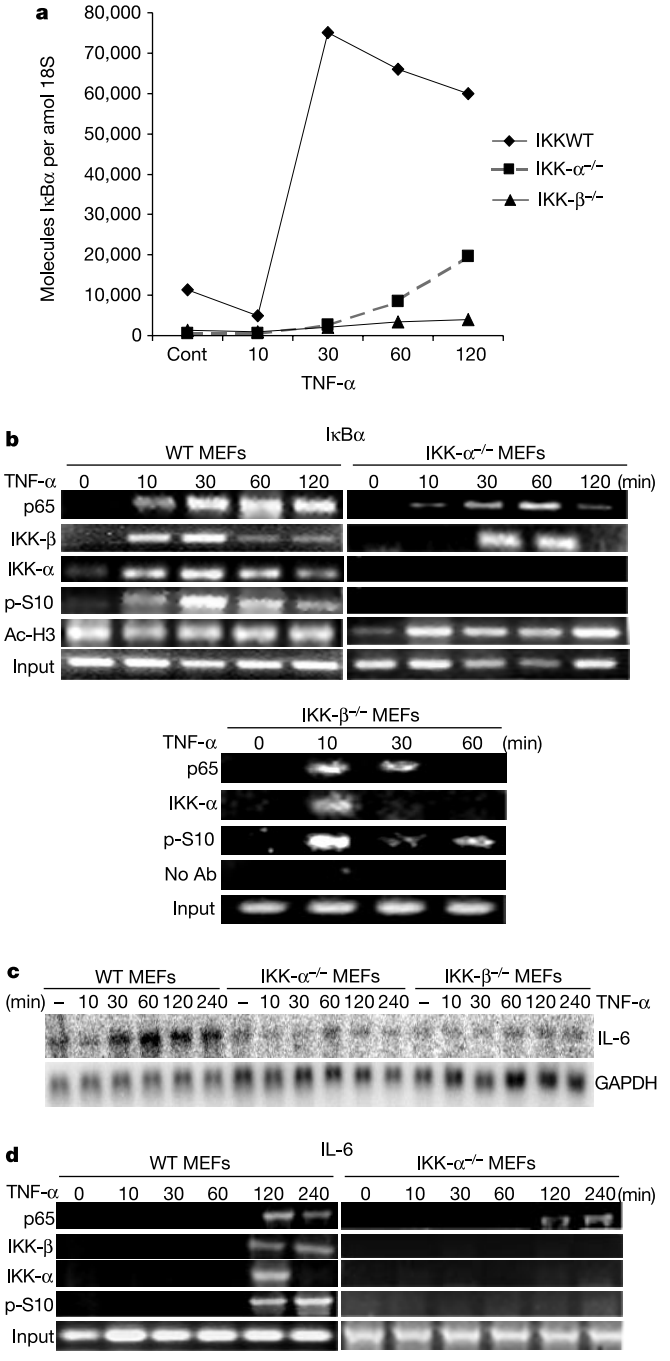

Figure 2 Promoter-associated IKK-α is essential for TNF-α-induced NF-κB-dependent gene expression and histone H3 phosphorylation. **a**, **c**, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ cells are defective in TNF-α-induced IκBα and IL-6 gene expression. IKK wild-type (WT), IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ MEFs were stimulated with TNF-α and RNA levels were measured by real-time PCR for IκBα (**a**) and northern blot analysis for IL-6 gene expression (**c**). **b**, **d**, Chromatin immunoprecipitation assays were performed on TNF-α-induced IKK WT (left panel), IKK-$\alpha^{-/-}$ (right panel), or IKK-$\beta^{-/-}$ MEFs (bottom panel) with p65, IKK-α, IKK-β, H3 Ser 10 phospho-specific or acetyl-H3 antibodies (AS). Associated DNA was analysed by PCR using IκBα (**b**) or IL-6 promoter-specific primers (**d**). These results are representative of three independent experiments.

To address whether IKK-α has a unique nuclear function in modulating gene expression, we examined the recruitment profile of p65 and the status of histone modifications at the IκBα promoter in IKK-$\alpha^{-/-}$ cells following TNF-α treatment. The kinetics of p65 recruitment in these cells is similar to those of wild-type MEFs, whereas IKK-β recruitment is delayed (Fig. 2b, right panel), indicating that p65 and IKK-β association with the IκBα promoter does not require IKK-α. The levels of TNF-α-induced p65 recruitment are reduced in IKK-$\alpha^{-/-}$ cells. Notably TNF-α-induced H3 Ser 10 phosphorylation levels are completely abolished in the IKK-$\alpha^{-/-}$ cells. Basal levels of H3 acetylation are decreased in the IKK-$\alpha^{-/-}$ cells but normal levels are restored after TNF-α-induction. These data indicate that IKK-α is required for Ser 10 phosphorylation of histone H3 at the IκBα promoter. The requirement for IKK-α in controlling H3 Ser 10 phosphorylation is also observed on another immediately accessible NF-κB-regulated gene, *Mip2* (also known as *Cxcl2*; data not shown).

Next, we investigated recruitment of p65 and IKK-α, and the status of H3 Ser 10 phosphorylation at the IκBα promoter, using IKK-$\beta^{-/-}$ MEFs. We detected reduced and transient levels of promoter-associated p65 and IKK-α after stimulation with TNF-α. The onset of Ser 10 phosphorylation matches the pattern of IKK-α recruitment in these cells after stimulation with TNF-α (Fig. 2b, lower panel). These data show that the loss of IKK-β does not affect Ser 10 phosphorylation at the IκBα promoter.

To examine further a requirement for IKK-α in regulating Ser 10 phosphorylation of histone H3, we evaluated the IL-6 gene, an NF-κB-regulated gene that requires prior chromatin modifications to ensure accessibility to NF-κB[11]. In wild-type MEFs, p65, IKK-α and IKK-β all show a similar profile of promoter recruitment occurring 2 h after stimulation with TNF-α (Fig. 2d, left panel). Similar to the results obtained with the IκBα promoter, the presence of IKK-α directly correlates with the onset of H3 Ser 10 phosphorylation. Promoter-associated IKK-α levels decrease 4 h after treatment whereas Ser 10 levels remain detectable, suggesting that either additional H3 kinases may be involved in the maintenance of Ser 10 levels at the IL-6 promoter or that Ser 10 phosphorylation levels remain stable after transient recruitment of IKK-α. Notably, we did not detect TNF-α-induced Ser 10 phosphorylation in IKK-$\alpha^{-/-}$ cells at the IL-6 promoter (Fig. 2d, right panel). p65 recruitment in IKK-$\alpha^{-/-}$ cells exhibits similar kinetics to wild-type cells, although overall levels appear to be lower. We did not detect IKK-β at this promoter in IKK-$\alpha^{-/-}$ cells, suggesting a role for IKK-α in recruiting IKK-β to the IL-6 promoter. Collectively, these results indicate a requirement for IKK-α in mediating H3 Ser 10 phosphorylation at different NF-κB-dependent promoters.

The timing of TNF-α-induced expression of IL-6 messenger RNA does not coincide with recruitment of p65 to the IL-6 promoter (Fig. 2c, d). We examined IL-6 mRNA levels in wild-type and p65$^{-/-}$ MEFs to determine whether expression of IL-6 is strictly p65-dependent. Northern blotting experiments revealed decreased IL-6 expression in p65$^{-/-}$ cells, although low-level induction of IL-6 by TNF-α is observed (data not shown). This is consistent with p65 being necessary for efficient IL-6 transcription but suggests that other transcription factors, probably additional NF-κB subunits (J.L.H., unpublished observations), can initiate IL-6 transcription at a minimal level after TNF-α stimulation.

The co-occupancy of p65 and IKK-α at both IκBα and IL-6 promoters in wild-type MEFs stimulated with TNF-α led us to examine whether p65 is required for the recruitment of IKK-α at these promoters. In p65$^{-/-}$ MEFs, neither IKK-α recruitment nor Ser 10 phosphorylation is detected, indicating that p65 activation

complexes are required for IKK-α association with NF-κB-regulated promoters (data not shown). Further investigation is needed to clarify whether p65 binding may function to alter the chromatin environment for IKK-α recruitment and subsequent Ser 10 phosphorylation of histone H3, or whether p65 directly recruits IKK-α in this response.

The role of IKK-α in controlling promoter-specific Ser 10 phosphorylation may be indirect through the regulation of other H3 kinases, or alternatively, IKK-α may directly phosphorylate histone H3. To address this latter point, we tested free core histones as substrates for recombinant IKK-α by *in vitro* kinase assays. IKK-α efficiently phosphorylates histone H3 *in vitro* in a dose-dependent fashion (Fig. 3a). Furthermore, H3 phosphorylation catalysed by IKK-α is comparable to levels obtained with known H3 kinases, MSK1 and RSK2 (refs 12, 13; Fig. 3a). Site-specificity for histone H3 phosphorylation by IKK-α was confirmed by protein immunoblotting with a well-characterized anti-H3 (Ser 10) phospho-specific antibody (Fig. 3b). IKK-β fails to phosphorylate H3 on free core histones after incubation with [γ-^{32}P]ATP *in vitro* or using the Ser 10 phospho-specific antibody (Fig. 3a, b). As a positive control for IKK activity, phosphorylation of glutathione *S*-transferase (GST)–IκBα fusion by IKK-α and IKK-β is shown (Fig. 3b, lower panel). The analysis of the histone H3 primary sequence does not reveal the IKK phosphorylation consensus sequence. These results demonstrate that IKK-α directly phosphorylates histone H3 protein on Ser 10 *in vitro*.

Our data demonstrate TNF-α-inducible IKK-α recruitment and subsequent Ser 10 phosphorylation on specific NF-κB-regulated promoters. To address the potential involvement of IKK-α in mechanisms associated with global levels of histone H3 phosphorylation, we took two approaches. First, we acid-extracted core histones from wild-type, IKK-α$^{-/-}$ and IKK-β$^{-/-}$ MEFs and examined H3 Ser 10 phosphorylation by western blotting. In wild-type and IKK-β$^{-/-}$ MEFs, TNF-α treatment leads to increased Ser 10 phosphorylation (Fig. 4). However, both basal and TNF-α-induced levels of Ser 10 phosphorylation are significantly reduced in IKK-α$^{-/-}$ cells (Fig. 4) but are restored by the introduction of stably expressed IKK-α (data not shown). Previous reports suggest that impaired recognition of phosphorylated Ser 10 by phospho-H3 antibodies may occur when adjacent lysine groups are acetylated[14,15]. Therefore, we tested an antibody against dimodified H3 to examine H3 phosphorylated on Ser 10 and acetylated on Lys 14. Levels of phosphorylated and acetylated H3 increase in response to TNF-α stimulation, with kinetics similar to that of Ser 10 phosphorylation in wild-type and IKK-β$^{-/-}$ MEFs (Fig. 4). However, levels of phosphorylated and acetylated H3 remain reduced in the IKK-α$^{-/-}$ MEFs. This suggests that the reduced affinity for Ser 10 phosphorylation in IKK-α$^{-/-}$ MEFs is not due to antibody occlusion by adjacent acetylation on Lys 14. In addition, global levels of acetylated H3 are largely unaffected in IKK-α$^{-/-}$ MEFs (data not shown). Second, immunofluorescence staining of asynchronous wild-type and IKK-α$^{-/-}$ cells with the same phospho-specific antibody demonstrates similar H3 Ser 10 phosphorylation levels in both cell types (data not shown). However, this probably represents mitotic-associated Ser 10 phosphorylation[16], not transcriptionally associated histone phosphorylation. Thus, although it is clear that IKK-α controls promoter-associated Ser 10 phosphorylation on a set of NF-κB-regulated genes, current investigation is aimed towards examining the role of IKK-α in modulating global levels of histone H3 phosphorylation.

Although a role for IKK-α in controlling NF-κB-dependent gene expression has been suggested previously, a mechanism whereby IKK-α might facilitate such a response has remained unclear. Our results suggest that one role for IKK-α in controlling gene expression is through an unexpected nuclear mechanism involving histone H3 phosphorylation. Furthermore, the data indicate that IKK-α directly phosphorylates histone H3 under TNF-α stimulation. However, we cannot rule out the possibility that IKK-α is involved in the regulation of other known H3 kinases. In this regard, we observe no TNF-α-induced changes in MSK1 or RSK2 protein levels in wild-type or IKK-α$^{-/-}$ MEFs (data not shown). Also, MSK1 activity is minimally induced by TNF-α stimulation in wild-type or IKK-α$^{-/-}$ MEFs using core histones as a substrate (data not shown). Therefore, our results suggest that IKK-α-mediated histone phosphorylation may provide one nucleosomal component in the overall mechanism required for optimal gene expression. Future experiments will be necessary to determine whether IKK-α-mediated Ser 10 phosphorylation regulates other histone modifications associated with positive gene expression, such as acetylation of Lys 14 on histone H3 as predicted by the histone code hypothesis[15,17–20].

Our observation that IKK-β is recruited to NF-κB-dependent promoters and is not required for histone phosphorylation (Fig. 2b, lower panel) indicates that IKK-α does not require IKK-β to control histone phosphorylation, and suggests a distinct chromatin-associated role for IKK-β in controlling NF-κB-dependent gene

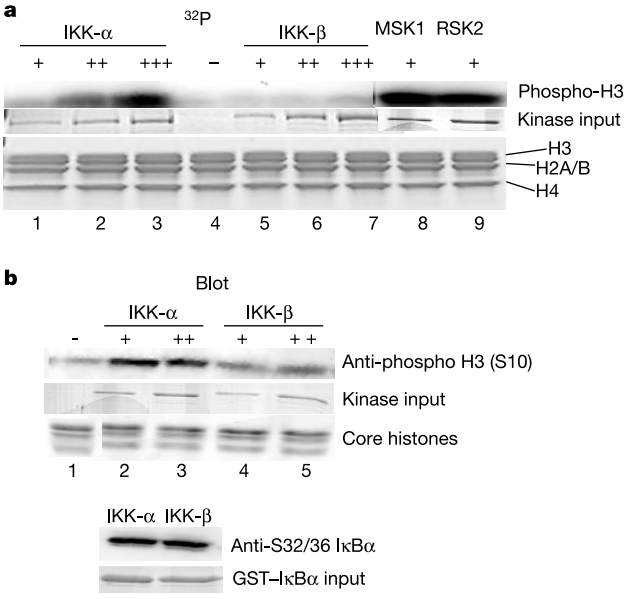

Figure 3 IKK-α directly phosphorylates histone H3 *in vitro*. **a**, Free core histones were incubated with increasing amounts of IKK-α or IKK-β (0.5, 1 and 2 μg, respectively) and [γ-^{32}P]ATP *in vitro* to analyse the dose response for histone H3 phosphorylation. For comparison, the activities of the H3 kinases (MSK1 (20 mU) and RSK2 (70 U)) towards the histones were included. Reactions were resolved by SDS–PAGE (15%) and analysed by autoradiography. Coomassie staining shows histones that were incubated with either IKK-α (lanes 1–3), no kinase (lane 4), IKK-β (lanes 5–7), MSK1 (lane 8) and RSK2 (lane 9). **b**, *In vitro* kinase assays were performed essentially as described in **a**, except that they were carried out in the absence (lane 1) or presence of IKK-α (lane 2, 3) and IKK-β (lanes 3, 4), and without radioactive ATP. Western blotting with anti-phospho-specific Ser 10 antibody indicates that IKK-α directly phosphorylates H3 on Ser 10 with a greater efficiency than IKK-β.

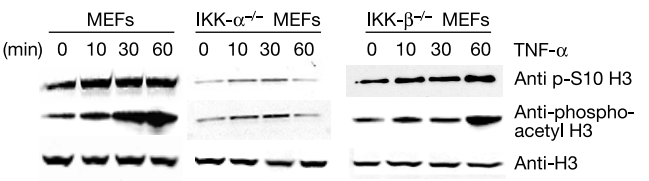

Figure 4 IKK-α modulates global levels of histone H3 phosphorylation. Acid-soluble proteins were extracted from asynchronous IKK wild-type, IKK-α$^{-/-}$ and IKK-β$^{-/-}$ MEFs, and were immunoblotted with anti-phospho-specific Ser 10, anti-phosphorylated/acetylated H3, or anti-H3 antibodies.

expression. Current experiments are directed towards investigating the role of promoter-localized IKK-β. Additionally, TNF-α induces recruitment of IKK-γ to the IκBα promoter, albeit at later time points than IKK-α and IKK-β in wild-type MEFs (data not shown). Collectively, these data support a function for IKK-α that is distinct from the classical role of the cytoplasmic IKK-α/β/γ complex in controlling cytokine-induced NF-κB-regulated gene expression. Thus, we propose independent roles for IKK-α, IKK-β and IKK-γ in cytokine-induced NF-κB-dependent gene expression. Whereas IKK-β controls IκBα degradation and efficient DNA binding of NF-κB subunits[1] and IKK-γ acts as a structural or regulatory mediator of this kinase complex[1,3,21], IKK-α functions as a chromatin modifier through histone phosphorylation. The data also raise the possibility of a role for IKK-α in NF-κB-independent gene expression. □

Methods

Cells and reagents

IKK wild-type, IKK-$\alpha^{-/-}$ and IKK-$\beta^{-/-}$ MEFs were provided by I. Verma and M. Karin. Antibodies against IKK-α, IKK-β, IKK-γ, p105/p50, actin and tubulin were obtained from Santa Cruz and Upstate Biotechnology. The p65/RelA-specific antibody was obtained from Rockland. Phosphorylated histone H3 (Ser 10) antibody was obtained from Cell Signal Technology. Acetylated (Lys 9/Lys 14) histone H3 antibody and phosphorylated/acetylated (Ser 10/Lys 14) histone H3 antibody was obtained from Upstate Biotechnology. TFIIB antibody was obtained from Transduction Laboratories. TNF-α (Kamiya) was used at a final concentration of 10 ng ml^{-1}.

Immunofluorescence

IKK wild-type, IKK-$\alpha^{-/-}$ or IKK-$\beta^{-/-}$ cells were seeded onto chamber slides (Nalge Nunc International) and treated with TNF-α (10 ng ml^{-1}) for 15 min. After fixation with 4% paraformaldehyde, cells were permeabilized with 0.2% Triton-X and blocked with 10% goat serum, 1% bovine serum albumin in phosphate-buffered saline (PBS). Primary antibodies were incubated at 4 °C overnight. One-hour incubations with fluorescein isothiocyanate-conjugated secondary antibodies (Santa Cruz Biotechnology) were used to detect primary antibody and were visualized on a Zeiss Axioskop. All images are shown at × 200.

Real-time quantitative PCR

Five micrograms of total RNA was incubated with Moloney murine leukaemia virus-reverse transcriptase (Invitrogen) as recommended by the manufacturer. The resulting complementary DNA was analysed quantitatively for the expression of IκBα by fluorogenic 5′-nuclease PCR as described previously. Specific primers (forward 5′-AGGATGAGCTGCCCTATGATGA-3′ and reverse 5′-TGCCACTTTCCACTTATAATGTCAGA-3′) and probe (5′-6-FAM TGTGTGTTTGGAGGCCA-TAMRA) were designed to the IκBα gene and PCR products were continuously measured by means of ABI Prism 7900 during 40 cycles. All data were normalized to 18S ribosomal RNA.

Northern blot analysis

Total RNA was isolated using Trizol (Invitrogen) as recommended by the manufacturer. A total of 10–20 μg total cellular RNA was separated on 1.5% formaldehyde-agarose gels and transferred overnight to a nylon filter according to standard procedures. RNA was then crosslinked to the membrane by ultraviolet irradiation (Stratagene) and probed with randomly labelled IL-6 probe. Hybridization and wash was preformed using ExpressHyb (Stratagene) as described by the manufacturer.

ChIP assay

ChIP analysis was performed following a protocol provided by Upstate Biotechnology under modified conditions. After TNF-α (20 ng ml^{-1}) stimulation, 3×10^6 cells were fixed with 1% formaldehyde. After 5 min, cells were washed extensively with ice-cold PBS and lysed for 10 min in lysis buffer (Upstate Biotechnology). Chromatin was sheared by sonication to an average size of approximately 1 kilobase and pre-cleared for 2 h at 4 °C with salmon sperm DNA-saturated protein G Sepharose. Chromatin solutions were precipitated overnight at 4 °C using 10 μl anti-p65, 10 μg anti-IKK-α or anti-IKK-β, 10 μl anti-phospho-H3 (Ser 10), and 5 μl acetylated H3-specific antibodies or beads alone. Immune complexes were collected with salmon sperm DNA-saturated protein G Sepharose for 1 h and washed extensively following the manufacturer's protocol. Input and immunoprecipitated chromatin were incubated at 65 °C overnight to reverse crosslinks. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNAs were analysed by PCR (30–35 cycles) using Platinum Taq PCR Master Mix (Invitrogen). The following promoter-specific primers were used: primer pair 5′-TGGCGAGGTCTGACTGTTGTGG-3′ and 5′-GCTCATCAAAAAGTTCCCTGTGC-3′ was used to amplify a 230-base-pair (bp) region in the mouse IκBα promoter; primer pair 5′-TGTGTGTGTGTGTATGTGTGTGTCG-3′ and 5′-TCGTTCTTGGTGGGCTCCAG-3′ was used to amplify a 440-bp region in the mouse IL-6 promoter or the β-actin promoter (5′-TGCACTGTGCGGCGAAGC-3′ and 5′-TCGAGCCATAAAAGGCAA-3′).

In vitro kinase assay

Kinase assays were performed following a previously described protocol (Upstate Biotechnology). Kinase activity was determined by incubating purified chicken core histones (10 mg ml^{-1}) with increasing amounts of recombinant IKK-α or IKK-β, or MSK1 (20 mU) and RSK2 (77 U) in the presence of 1 μCi ml^{-1} [γ-^{32}P]ATP or cold ATP (100 μM) for 30 min at 30 °C. Reactions were resolved by SDS–polyacrylamide gel electrophoresis (PAGE; 15%) and processed for autoradiography or protein immunoblotting. Recombinant IKK-α and IKK-β were provided by L. Dang.

Western blot analysis

Extractions of acid-soluble proteins were done according to the protocol described by Upstate Biotechnology and resolved on 10–20% Tris-tricine SDS–PAGE gels.

Received 6 March; accepted 28 March 2003; doi:10.1038/01648.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank D. Kashatus for critical reading of the manuscript. We appreciate scientific discussions with Y. Yamamoto and R. Gaynor. Support was provided by the NIH and by the UNC breast cancer SPORE program to A.S.B., and by the UNC Comprehensive Center for Inflammatory Disorders.

Competing interests statement The authors declare that they have no competing financial interests.

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