Positive and Negative Regulation of IκB Kinase Activity Through IKKβ Subunit Phosphorylation

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IκB [inhibitor of nuclear factor κB (NF-κB)] kinase (IKK) phosphorylates IκB inhibitory proteins, causing their degradation and activation of transcription factor NF-κB, a master activator of inflammatory responses. IKK is composed of three subunits—IKKα and IKKβ, which are highly similar protein kinases, and IKKγ, a regulatory subunit. In mammalian cells, phosphorylation of two sites at the activation loop of IKKβ was essential for activation of IKK by tumor necrosis factor and interleukin-1. Elimination of equivalent sites in IKKα, however, did not interfere with IKK activation. Thus, IKKβ, not IKKα, is the target for pro-inflammatory stimuli. Once activated, IKKβ autophosphorylated at a carboxyl-terminal serine cluster. Such phosphorylation decreased IKK activity and may prevent prolonged activation of the inflammatory response.

In unstimulated cells, transcription factor NF-κB (*1*), is kept in the cytoplasm through interaction with the IκB inhibitory proteins (*2*). Exposure to proinflammatory stimuli, such as tumor necrosis factor (TNF), results in phosphorylation, ubiquitilation, and then degradation of IκB. Liberated NF-κB dimers are translocated to the nucleus, where they activate transcription of target genes. The protein kinase complex that phosphorylates IκBs in response to proinflammatory signals contains two catalytic subunits, IKKα and IKKβ (or IKK1 and IKK2) (*3–6*), and a regulatory subunit, IKKγ [or NEMO (NF-κB essential modulator)] (*7, 8*). IKK activity is rapidly stimulated upon exposure of cells to proinflammatory stimuli. Two candidate IKK kinases are NF-κB–inducing kinase (NIK) (*9, 10*) and MAP kinase kinase kinase-1 (MEKK1) (*11, 12*), but their physiological roles are not clear (*13*). NIK preferentially phosphorylates IKKα (*10*), whereas MEKK1 preferentially phosphorylates IKKβ (*12*). Mutations within the activation or T loops of either IKKα or IKKβ were reported to prevent kinase activation or generate constitutively active IKKs (*4, 10*).

We studied the control of IKK phosphorylation and activity in TNF-stimulated cells and obtained evidence that IKK activity is activated through T loop (activation loop) phosphorylation of IKKβ, but not IKKα. Once activated, IKKβ undergoes progressive autophosphorylation at multiple serines located next to its COOH-terminus. This phosphorylation decreases kinase activity and contributes to the transient nature of IKK activation.

We incubated HeLa cells with [^{32}P]orthophosphate, stimulated them with TNF, and isolated the IKK complex at various time points (*14*). Although activity of IKK was stimulated within 2 min, the earliest detectable increase in its phosphorylation, affecting all three subunits, occurred at 10 min (Fig. 1A) (*15*). After 15 to 20 min, IKK activity declined faster than its phosphorylation (Fig. 1A). Phosphoamino acid analysis (*16, 17*) indicated that IKKα and IKKβ were phosphorylated on serines (*18*). Because IKKβ, but not IKKα, functions in IKK activation (see below), we concentrated on identification of its phosphoacceptor sites. IKKβ was digested with cyanogen bromide (CNBr) or endopeptidase LysC, and the resulting peptides were separated on Tris-Tricine gels (*16*). Most labeling occurred on a 7.5-kD

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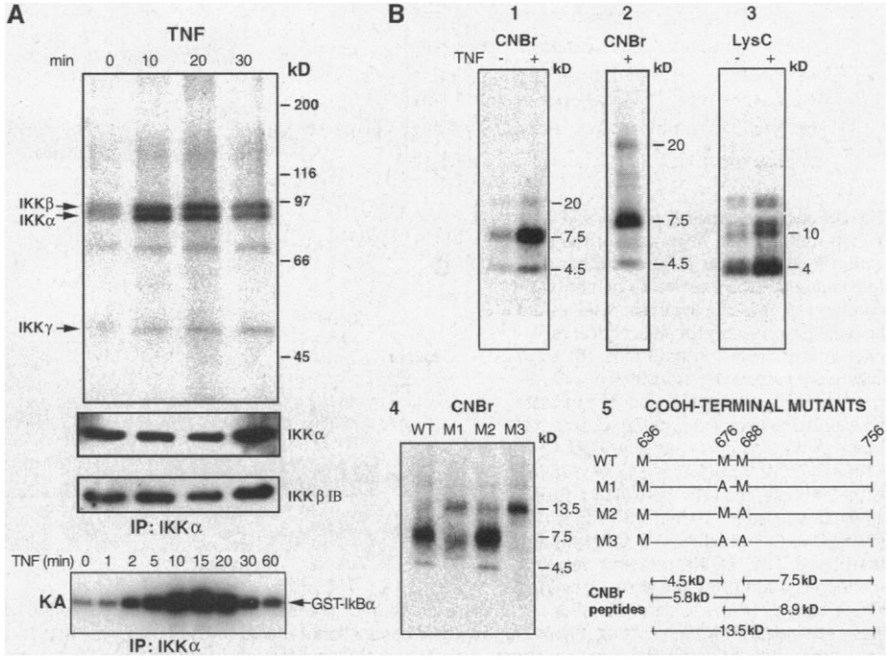

Fig. 1. Biochemical analysis of IKK phosphorylation. **(A)** HeLa cells incubated for 5 hours with [^{32}P]orthophosphate were stimulated with TNF (20 ng/ml) for the indicated times, then lysed (*14*). The IKK complex was immunoprecipitated (IP) with antibody to IKKα (*3*), resolved by gel electrophoresis and transferred onto a membrane. Phosphoproteins were detected by autoradiography. IKKα and IKKβ were identified by immunoblotting (IB). To measure activation kinetics, IKK was isolated by immunoprecipitation with anti-IKKα from TNF-stimulated HeLa cells and its activity was measured by phosphorylation of GST-IκBα(1-54) as described (*3*). **(B)** Phosphopeptide mapping of IKKβ from HT-29 cells stably expressing HA-tagged IKKβ. Cells were incubated with [^{32}P]orthophosphate and TNF-stimulated for 10 min as in (A). After immunopurification, the HA-IKKβ band was digested with CNBr or endopeptidase LysC as described (*16*). Peptides were resolved on Tris-Tricine gels (20% in panels 1, 3, and 4; 16.5% in panel 2), and phosphopeptides were detected by autoradiography. To confirm correct positioning of the COOH-terminal CNBr phosphopeptides, we generated mutants M1 (M676A), M2 (M688A), and M3 (MM676/688AA) in which the two COOH-terminal methionines (M) were replaced with alanines (A). HA-tagged wt and mutant IKKβs were transiently expressed in HT-29 cells (*3*) and subjected to phosphopeptide mapping as described above. The actual and predicted CNBr peptides are depicted in panels 4 and 5, respectively.

CNBr-digested or a 4-kD LysC-digested peptide (Fig. 1B). CNBr cleavage also generated 20- and 4.5-kD phosphopeptides. The 7.5-kD CNBr peptide probably is a COOH-terminal fragment generated by cleavage at M688 (*19*), whereas the 4.5-kD phosphopeptide is generated by cleavage at M636 and M676. The 4-kD LysC fragment represents cleavage at K665 and K703 or K704. To confirm these assignments, we mutated M676 and M688 to alanines. These mutations did not interfere with IKKβ phosphorylation but altered the CNBr cleavage pattern (Fig. 1B). Therefore, most of IKKβ phosphorylation occurs between M636 and E700, a region which contains nine serines.

Based on its size, the 20-kD CNBr phosphopeptide encompasses the T loop of IKKβ. The T loops of IKKα and IKKβ contain two conserved serines in positions similar to the activating sites of MEK1 and MEK2 (Fig. 2A). Conversion of these serines to alanines on either IKKα or IKKβ was reported to interfere with IKK activation (*4, 10*). Conversion of both serines in IKKβ to glutamates generated a constitutively active kinase IKKβ(EE) that was found to still be phosphorylated at the COOH-terminal sites after CNBr digestion, but no longer at the 20-kD T loop peptide (see below). We compared the relative contribution of the T loop serines of IKKα and IKKβ to IKK activation. Whereas the $Ser^{177} \rightarrow Ala^{177}$ (S177A) mutation in IKKβ slightly decreased cytokine responsiveness, the S181A mutation had a more severe effect, and the replacement of both sites abolished IKK activation altogether (Fig. 2B). The equivalent mutations in IKKα had no effect on IKK activation. Similar results were obtained when the responses of the IKKα and IKKβ mutants to coexpressed NIK and MEKK1 were examined (Fig. 2C) (*15*). S176A and S180A mutations in IKKα were reported to interfere with its activation by NIK (*10*). This discrepancy may reflect differences in transfection conditions (*20*). Under our conditions, the epitope-tagged IKK subunits are not overexpressed and are incorporated into functional cytokine-responsive 900-kD IKK complexes (*3, 8*). Therefore, within the IKK complex, phosphorylation of the IKKβ T loop is more important for activation by cytokines than phosphorylation of the homologous sites in IKKα. These results are consistent with those obtained from analysis of IKKα- and IKKβ-knockout mice (*21, 22*).

We examined the effect of the T loop mutations on phosphorylation of IKKα and IKKβ in response to NIK expression. Coexpression of wild-type (wt) NIK, but not a catalytically inactive mutant [NIK(AA)], enhanced phosphorylation of IKKα and IKKβ (Fig. 3A). In the case of IKKβ, most of this increase was due to autophosphorylation, because NIK had no effect on phosphorylation of catalytically inactive IKKβ(K44A). However, NIK did enhance the phosphorylation of an IKKα(K44M) mutant (Fig. 3A). Similar results were interpreted to suggest that IKKα is a better substrate for NIK than is IKKβ (*10*). However, we expect that residual NIK-induced phosphorylation of IKKα (K44M) is due to activation of endogenous IKKβ that associates with the transiently expressed IKKα (*3*).

Substitution of either of the activating phosphoacceptor sites of IKKα with alanines reduced the extent of NIK-induced phosphorylation (*15*), but even the double mutant IKKα(AA) was still phosphorylated in response to NIK (Fig. 3B). In the case of IKKβ, however, the S177A mutation resulted in a partial decrease in NIK-induced phosphorylation, whereas the S181A mutation had a more substantial effect (*15*) and the double mutant IKKβ(AA) was no longer phosphorylated (Fig. 3B). Thus, for IKKβ, but not IKKα, there is full correspondence between the effect of T loop mutations on NIK-induced phosphorylation and IKK activation.

Next, we examined the role of the clustered COOH-terminal phosphorylation sites. Because the inactivating (K44A) and the T loop (S177A and S181A) mutations completely prevented IKKβ phosphorylation (Fig. 3), it is likely that the COOH-terminal cluster is autophosphorylated. To identify which of the serines are indeed phosphorylated and determine their effect on IKKβ activity, we replaced them with alanines (Fig. 4A). Substitution of a few serines at a time modestly decreased IKKβ phosphorylation (*15*), but the simultaneous substitution of 10 serines, located between position 670 and position 705, had a substantial effect (Fig. 4B). Phosphopeptide mapping confirmed that the

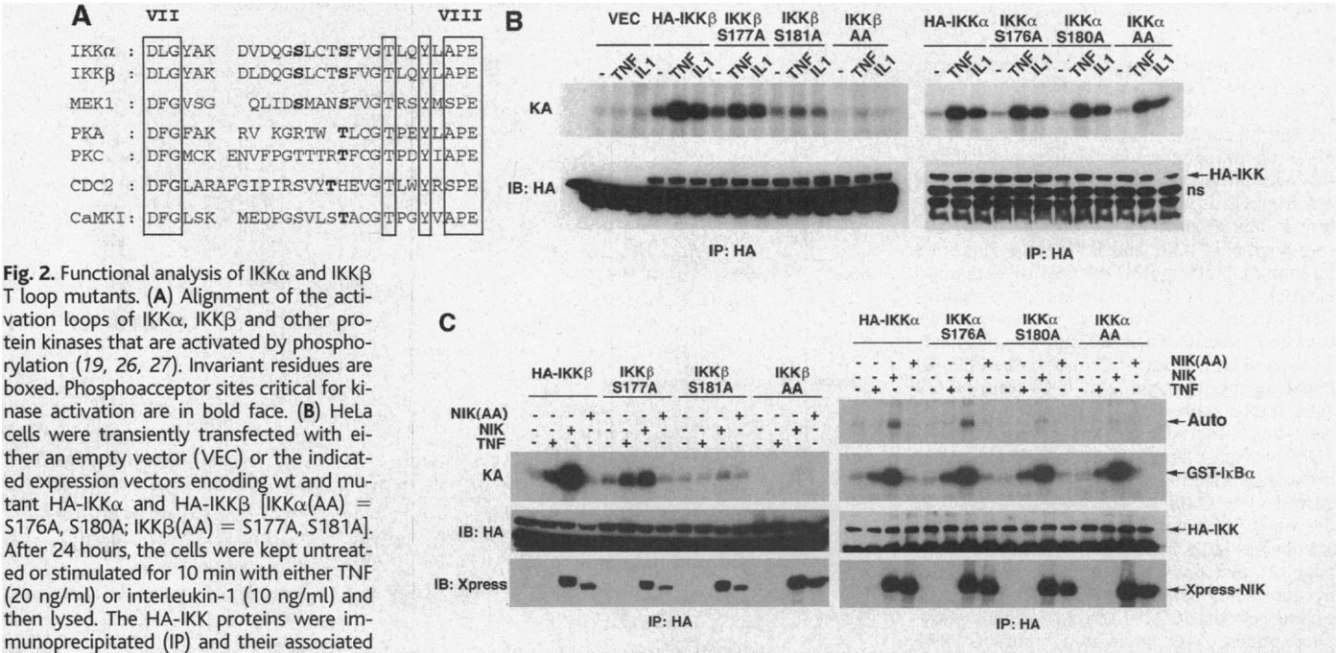

Fig. 2. Functional analysis of IKKα and IKKβ T loop mutants. (**A**) Alignment of the activation loops of IKKα, IKKβ and other protein kinases that are activated by phosphorylation (*19, 26, 27*). Invariant residues are boxed. Phosphoacceptor sites critical for kinase activation are in bold face. (**B**) HeLa cells were transiently transfected with either an empty vector (VEC) or the indicated expression vectors encoding wt and mutant HA-IKKα and HA-IKKβ [IKKα(AA) = S176A, S180A; IKKβ(AA) = S177A, S181A]. After 24 hours, the cells were kept untreated or stimulated for 10 min with either TNF (20 ng/ml) or interleukin-1 (10 ng/ml) and then lysed. The HA-IKK proteins were immunoprecipitated (IP) and their associated kinase activity (KA) was determined as in Fig. 1A. Expression of HA-IKKα/β was examined by immunoblotting (IB); ns, none specific band. All procedures were as described (*3*). (**C**) The same constructs were transiently transfected into HEK-293 cells either alone or together with expression vectors for Xpress-tagged NIK or its catalytically inactive mutant NIK(AA). After 24 hours, the cells were left untreated or stimulated for 10 min with TNF, lysed, and the kinase activity (KA) of the HA-IKK proteins was measured. Autophosphorylation (Auto) of wt and mutant HA-IKKα was determined by kinase assays done in the absence of extraneous substrate. The amounts of HA-IKK and Xpress-NIK were examined by immunoblotting (IB).

4.5-kD CNBr peptide represents phosphorylation at S670 and S672, whereas the 7.5-kD CNBr phosphopeptide contains nine serines; the substitution of five of them with alanines (as in M10) does not completely eliminate its phosphorylation (Fig. 4C). Both phosphopeptides are strongly and constitutively phosphorylated in the IKKβ(EE) mutant. To examine the effect of COOH-terminal autophosphorylation on IKKβ activity, we stably expressed hemagglutinin (HA)-tagged IKKβ-M10 (HA-IKKβ-M10) and wt HA-IKKβ in HeLa cells. Whereas wt HA-IKKβ was transiently activated by TNF with kinetics identical to those of endogenous IKK, HA-IKKβ-M10 had higher basal activity, and its TNF-induced activation lasted at least four times longer than that of wt enzyme (Fig. 4D). Identical results were obtained with two other mutants, M12 and M14 (Fig. 4A) (*15*). To further establish the negative regulatory role of the COOH-terminal autophosphorylation cluster, the same 10 serines mutated in IKKβ-M10 were converted to glutamates (abbreviated as S10E), and the resulting mutant IKKβ-S10E was stably expressed in HeLa cells. Indeed, the phosphomimic mutation obliterated most of the response to TNF (Fig. 4D).

The COOH-terminal autophosphorylation sites are located next to the helix-loop-helix

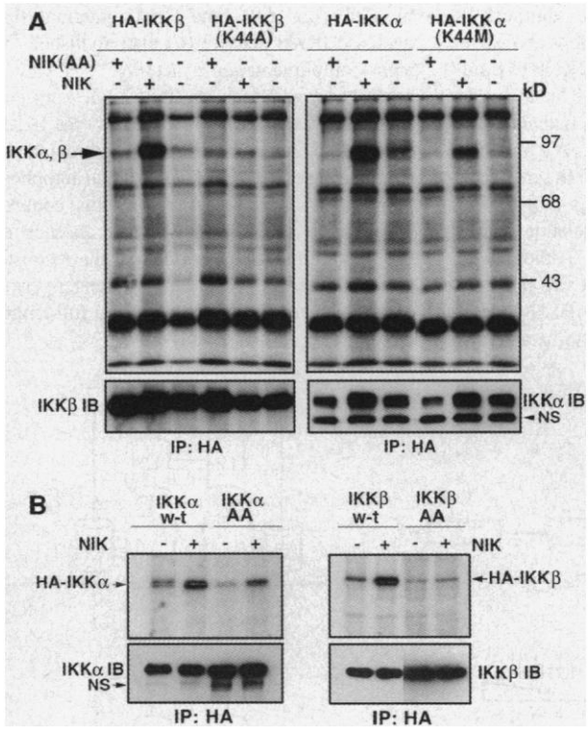

Fig. 4. In vivo phosphorylation of wt and mutant IKKα and IKKβ. (**A**) HA-tagged wt and catalytically inactive forms of IKKα and IKKβ were transiently expressed in HEK-293 cells without or with wt or catalytically inactive NIK. After 24 hours, the cells were incubated with [^{32}P]orthophosphate, and the HA-IKK proteins were immunopurified and analyzed as in Fig. 1A. IKKα and IKKβ expression was determined by immunoblotting (IB). (**B**) wt HA-IKKα, HA-IKKβ and their T loop mutants were transiently expressed without or with NIK. The cells were incubated with [^{32}P]orthophosphate. The HA-IKK proteins were isolated and analyzed as described above.

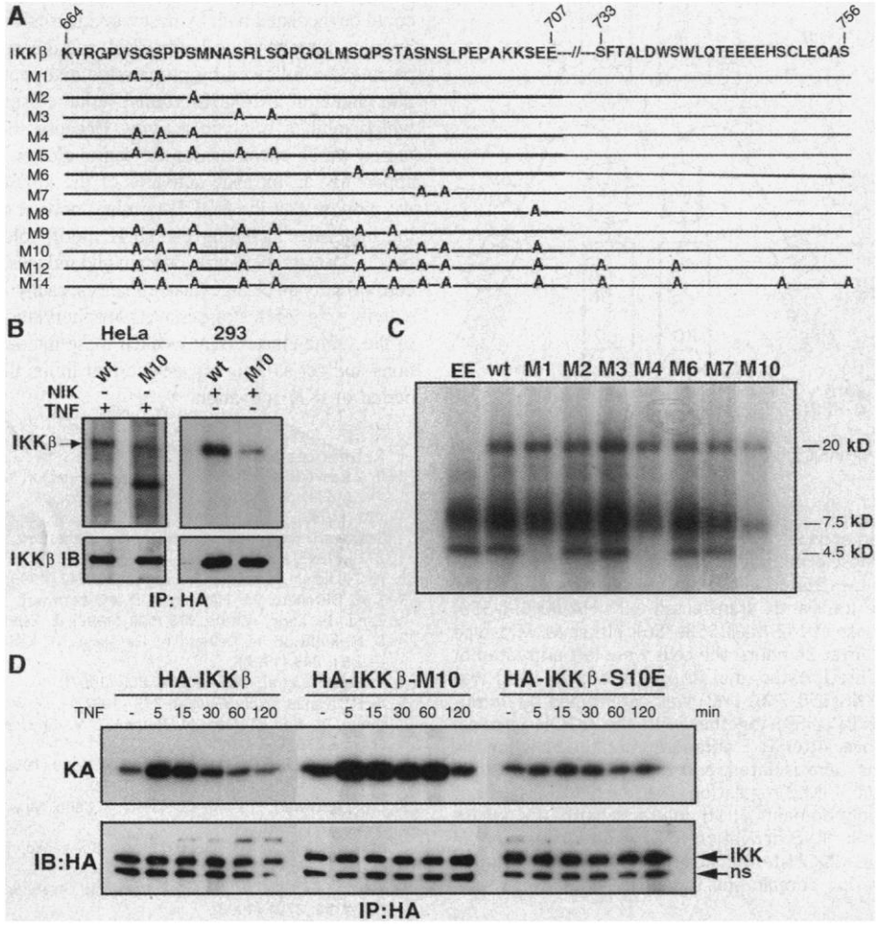

Fig. 3. Negative regulation of IKKβ activity by COOH-terminal autophosphorylation. (**A**) Amino acid sequence of the COOH-terminal serine cluster of IKKβ and the different alanine substitution mutants (M1 to M14). (**B**) wt HA-IKKβ or M10 were expressed stably in HeLa cells or transiently together with NIK in HEK-293 cells. The ^{32}P-labeled proteins were isolated and analyzed as described (Fig. 1A). (**C**) wt and mutant versions of IKKβ (EE = S177E, S181E) were expressed with NIK. The ^{32}P-labeled proteins were cleaved with CNBr and the resulting peptides were separated as in Fig. 1B. (**D**) Kinetics of wt IKKβ, IKKβ-M10 and IKKβ(S10E) activation in stably transfected HeLa cells. The HA-tagged IKKβ proteins were isolated at the indicated time points after TNF addition and their IKK activity and expression were determined as described (Fig. 2B).

(HLH) motif, mutations in which diminish kinase activity (*6, 15*). HLH mutations abolish the activity of purified recombinant IKKβ (*6*), suggesting that this motif may serve as an endogenous activator of IKKβ, akin in function to the cyclin subunits of CDKs (*23*). We therefore tested whether a COOH-terminal fragment containing the HLH motif (residues 558 to 756) activated in *trans* an IKKβ deletion mutant lacking this region (Fig. 5A). Although the COOH-terminal deletion mutant was practically inactive, coexpression of the HLH-containing fragment restored its ability to be activated by TNF (Fig. 5B). The HLH mutations that decrease IKKβ activity (*6*) also abolished the *trans*-complimentation activity. However, when expressed in *trans*, the COOH-terminal fragment was not phosphorylated by the IKKβ kinase domain (*15*), and therefore, we could not examine the effect of COOH-terminal autophosphorylation on IKK activity in this context. Nevertheless, consistent with the absence of COOH-terminal phosphorylation, the reconstituted IKKβ remained active for a longer period after TNF stimulation than did the full-length enzyme (Fig. 5C).

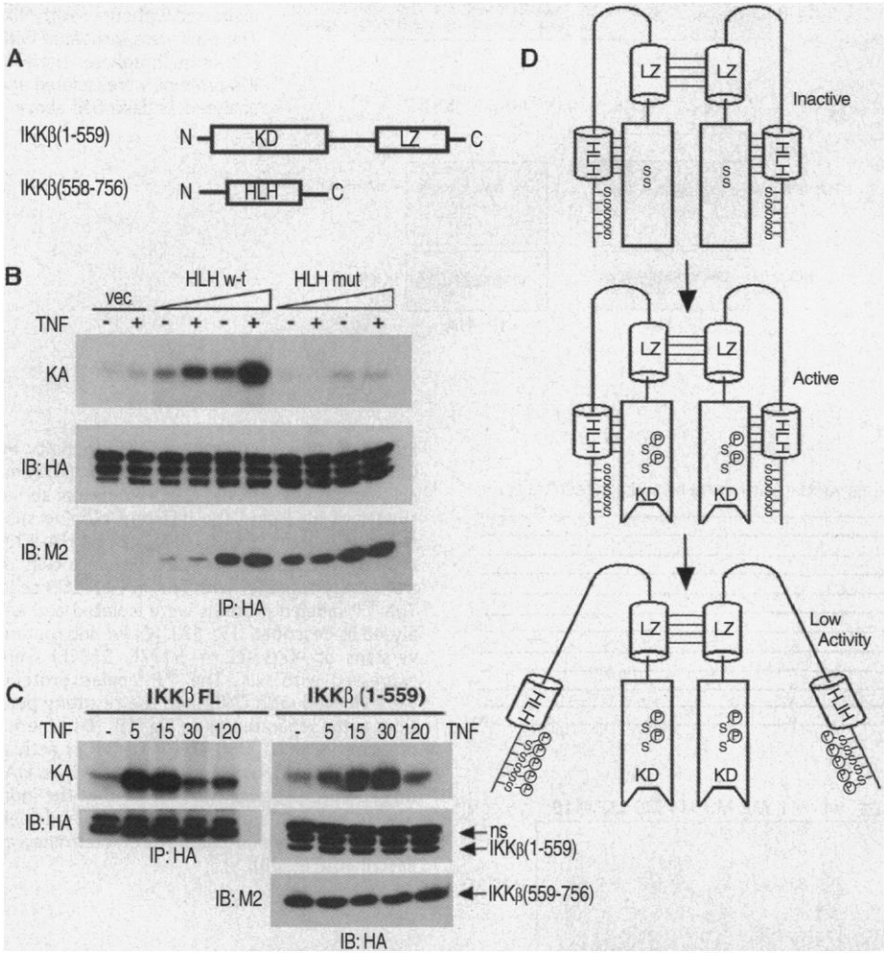

Fig. 5. The COOH-terminal region of IKKβ acts as an activator of its kinase domain. (**A**) Schematic representation of IKKβ truncation mutants used in these experiments. Amino acids 1 to 559 include the kinase domain (KD) and leucine zipper (LZ); residues 558 to 756 contain the HLH motif and the COOH-terminal serine cluster. (**B**) HeLa cells were transiently transfected with HA-IKKβ(1-559) together with empty vector (vec) or various amounts of M2-IKKβ(558-756), either as wild-type (wt) or with mutations (mut) (*6*) in the HLH motif. After 24 hours, the cells were left untreated or stimulated with TNF, HA-IKK(1-559) was immunoprecipitated, and its kinase activity (KA) was determined. Expression of IKKβ(1-559) (HA) and IKKβ(558-756) (M2) was determined by immunoblotting. (**C**) Full-length (FL) HA-IKKβ or HA-IKKβ(1-559) together with the COOH-terminal domain, M2-IKKβ(558-756), were expressed as above. After TNF stimulation of the cells for the indicated times (in minutes), the HA-IKKβ proteins were isolated, and their kinase activity and amounts were determined as described. (**D**) A model of IKKβ regulation. The inactive kinase is not phosphorylated, and the COOH-terminal activation domain (HLH) interacts with the kinase domain (KD). Phosphorylation of the T loop results in IKKβ activation followed by its sequential COOH-terminal autophosphorylation. When 9 or 10 COOH-terminal serines are phosphorylated, the interaction between the COOH-terminal activation domain and the kinase domain is weakened, and activity of IKKβ decreases.

Our results indicate that only IKKβ phosphorylation contributes to IKK activation by proinflammatory cytokines or by cotransfected NIK and MEKK1. We found that IKKα is not required for stimulation of IKK activity, a conclusion consistent with the genetic analysis of IKK function (*21, 22*). Whereas disruption of the *IKK*α locus has no effect on IKK activation and IκB degradation in response to proinflammatory stimuli (*21*), disruption of the *IKK*β locus results in a major defect in both events (*22*). It is likely, however, that when associated with IKKβ, IKKα is activated by the former and may contribute to total IKK activity, as it is capable of direct IκB phosphorylation (*6*).

IKKβ is also the site for negative regulation of IKK activity. Autophosphorylation of a serine cluster located between the HLH motif of IKKβ and its COOH-terminus decreases IKK activity and contributes to its transient activation in TNF-stimulated cells. A similar mechanism may affect IKKα. Because of the positive autoregulatory nature of the NF-κB signaling pathway and the potential toxicity and pathophysiology associated with its prolonged activation (*24*), it is important not only to rapidly activate this system in response to infection but also to decrease its activity once the infectious challenge disappears. IKKβ is rapidly activated through phosphorylation at its T loop [which could be mediated both by upstream kinases and by trans-autophosphorylation (*25*)], and this appears to be followed by progressive autophosphorylation at a COOH-terminal serine cluster, which inhibits catalytic activity. Because this region which encompasses the serine cluster is apparently an intrinsic activator of the kinase, we propose that the COOH-terminal portion of the molecule, including the HLH motif, folds back to contact the kinase domain and induces a conformational change that enhances catalytic activity (Fig. 5D). Progressive phosphorylation of the serine cluster may weaken these interactions and act as a timing device that limits the period of IKK activation.

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14. Cells incubated with [^{32}P]orthophosphate (2 mCi/ml) for 5 hours were TNF-stimulated, washed, and harvested in lysis buffer. IKK was immunoprecipitated from precleared lysates using anti-IKKα as described (3). Immune complexes were washed with lysis buffer containing 2 M urea. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (7.5%) and transferred to Immobilon-P membrane (Millipore) for autoradiography and immunoblot analysis. IKK assays were performed as described (3).
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19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Limb and Skin Abnormalities in Mice Lacking IKKα

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The gene encoding inhibitor of kappa B (IκB) kinase α (IKKα; also called IKK1) was disrupted by gene targeting. IKKα-deficient mice died perinatally. In IKKα-deficient fetuses, limb outgrowth was severely impaired despite unaffected skeletal development. The epidermal cells in IKKα-deficient fetuses were highly proliferative with dysregulated epidermal differentiation. In the basal layer, degradation of IκB and nuclear localization of nuclear factor kappa B (NF-κB) were not observed. Thus, IKKα is essential for NF-κB activation in the limb and skin during embryogenesis. In contrast, there was no impairment of NF-κB activation induced by either interleukin-1 or tumor necrosis factor–α in IKKα-deficient embryonic fibroblasts and thymocytes, indicating that IKKα is not essential for cytokine-induced activation of NF-κB.

The IκB kinase, a large protein complex, phosphorylates two serine residues of the IκB proteins. This leads to degradation of IκB and activation of NF-κB transcription factors (*1*). IKKα was identified as a subunit of the IκB kinase complex that directly phosphorylates IκB (*2, 3*). IKKβ was subsequently identified as a second subunit of the IκB kinase complex that forms a heterodimer with IKKα (*3, 4*). In vitro studies have indicated that both IKKα and IKKβ (also called IKK2) may contribute to tumor necrosis factor–α (TNF-α)– and interleukin-1 (IL-1)–induced activation of NF-κB (*2–4*).

To assess the in vivo role of IKKα, we disrupted the *IKK*α gene by homologous recombination in E14.1 embryonic stem (ES) cells (*5*). A targeting vector was constructed to replace an exon encoding subdomain VI of the kinase catalytic portion with a neomycin resistance gene. Two correctly targeted ES clones successfully transmitted the disrupted allele through the germ line (Fig. 1A). The heterozygous (IKKα$^{+/-}$) mice were phenotypically normal and healthy. To generate IKKα$^{-/-}$ mice, the heterozygotes were crossed. IKKα$^{-/-}$ progeny were born with abnormal appearance and died within 4 hours after birth. Newborn IKKα$^{-/-}$ pups had defective development of limbs and tails (Fig. 1D), and their skin was abnormally shiny. Northern (RNA) and protein immunoblot analysis of embryonic fibroblast (EF) cells confirmed that the disruption of the *IKK*α gene abolished the expression of IKKα mRNA and protein (Fig. 1, B and C). Expression of mRNA and protein for IKKβ was slightly increased in IKKα$^{-/-}$ EF cells.

Examination of stained skeletal preparations from the fetus at 18.5 days postcoitum (dpc) revealed that IKKα$^{-/-}$ mice had no defect in development of bone or cartilage, although the lengths of limb, tail, and craniofacial bones and cartilage were shorter than those for wild-type animals (Fig. 1E). Leg bones were compactly and tightly folded and tail cartilage was rolled up in IKKα$^{-/-}$ pups. These findings indicate that skeletal development was normal; however, limb mesenchyme outgrowth was impaired in IKKα$^{-/-}$ fetuses. Activation of NF-κB is essential for limb development in chickens (*6*). Therefore, we analyzed whether IKKα was expressed in the developing limb by whole-mount in situ hybridization (*7*). *IKK*α was expressed predominantly in the limb buds of wild-type fetuses at 12.5 dpc (Fig. 2A). In IKKα$^{-/-}$ fetuses, *IKK*α was not expressed, and the limb bud showed a slightly abnormal phenotype relative to that of wild type (Fig. 2B). *IKK*β was also expressed in the limb buds, particularly the forelimbs of wild-type as well as IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2, C and D). A *Drosophila melanogaster* homolog of NF-κB, Dorsal, positively and negatively regulates expression of *twist* and *decapentaplegic* (*dpp*), respectively (*8*). The murine *twist* homolog is expressed in limb bud mesenchyme (*9*), and mutations in *TWIST* lead to craniofacial and limb anomalies in humans (*10*). In the wild-type fetuses, *Twist* was expressed in the limb buds at 12.5 dpc (Fig. 2E). However, expression of *Twist* was reduced in the limb buds of IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2F). Expression of the bone morphogenic protein-4 gene (*BMP4*), the vertebrate *dpp* homolog, was not altered in the limb buds of IKKα$^{-/-}$ fetuses at 12.5 dpc (Fig. 2, G and H). Reduced *Twist* expression in IKKα$^{-/-}$ fetuses was also observed at 13.5 dpc (*11*). Taken together, these results indicate that IKKα regulates gene expression required for limb development, possibly through activation of NF-κB.

Tissue sections of skin at 18.5 dpc were stained with hematoxylin and eosin and examined by light microscopy. At this developmental stage, the full program of epidermal differentiation was nearly complete in wild-type mice (Fig. 3A). In contrast with the ridged and laminated normal stratum corneum of wild-type mice, IKKα$^{-/-}$ mice exhibited prominent parakeratosis without a visible stratum granulosum (Fig. 3B). The stratum spinosum of IKKα$^{-/-}$ epidermis was hyperplastic. The development

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