ed to nanoelectrospray mass spectrometry (25). The peptide mixture was micropurified on a capillary containing 50 nl of Poros R2 resin (Perseptive Biosystems, Framingham, MA). The peptides were washed and then step-eluted with 0.5 μl of 50% MeOH in 5% formic acid into a nanoelectrospray needle. This needle was transferred to an APIII mass spectrometer (Perkin-Elmer, Sciex, Toronto, Canada) and the sample was sprayed for about 20 min. During this time, peptide ions apparent from the mass spectrum were selected and isolated and then fragmented in the collision chamber of the mass spectrometer. From the tandem mass spectra, short stretches of sequence were assembled into peptide sequence tags (18) and compared with a protein sequence database or an EST database by using PeptideSearch. Three peptides matched the IKK-1 sequence. A1, IIDLGYAK; A2, VEVALSNIK; A3, SIQLDLER. Three other peptides matched human EST sequences in the EST database: B1, ALELLPK; B2, VIYTQLSK; B6, LLLQAIQSFEK. These three sequences all match EST clone AA326115. The peptide B4 with the sequence LGTGGFGNVIR was found in clone R06591. After the full-length IKK-2 sequence was obtained (19), two more peptides (B3, ALDDILNLK; B5, DLKPENIVLQQGEQR) were found in the sequence. Peptide A1 is present in both IKK-1 and IKK-2 sequences.

16. A. Shevchenko *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14440 (1996).
17. M. A. Connely and K. B. Marcu, *Cell. Mol. Biol. Res.* **41**, 537 (1995).
18. M. Mann and M. S. Wilm, *Anal. Chem.* **66**, 4390 (1994); M. Wilm *et al.*, *Nature* **379**, 466 (1996); M. Mann, *Trends Biol. Sci.* **21**, 494 (1996).

19. Several peptides were identical matches to human EST clones. All the EST clones were similar to human and mouse CHUK-1 (IKK-1). These clones were obtained (Genome Systems, Inc.) and the precise nucleotide sequence was determined and used to design primers to clone human IKK-2 by polymerase chain reaction (PCR) from a human HeLa cell cDNA library (Clontech, Inc.). Several IKK-2 cDNA clones were isolated and sequenced. Full-length mouse IKK-1 and a partial human IKK-1 nucleotide sequence were available in the comprehensive database. Primers were designed to clone by PCR the human and mouse IKK-1 cDNAs. The partial human IKK-1 coding region was used to probe a HeLa cDNA phage library (Stratagene) to obtain the full-length human IKK-1 cDNA clone by standard procedures.
20. For in vitro translation studies, HA-tagged IKK-1 and Flag-tagged IKK-2 were in vitro translated in RRLs, either separately or alone, exactly as described in the manufacturer's protocol (Promega).
21. C.-F. Zheng and K.-L. Guan, *EMBO J.* **13**, 1123 (1994).
22. For immunocytochemistry, HeLa cells were transiently transfected with either HA-tagged IKK-1 or Flag-tagged IKK-2 as indicated. Cells were washed with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min, and permeabilized with wash buffer (0.5% Triton X-100, 0.01% azide in PBS). Cells were blocked with wash buffer containing donkey serum (5%) (Jackson Immunoresearch Laboratories) and probed with primary antibody—anti-RelA (polyclonal antibody), 1:2000 (Santa Cruz); anti-HA (monoclonal antibody), 1:1000 (Eastman Kodak); or anti-Flag (monoclonal antibody), 1:1000 (Babco)—followed by secondary antibody—donkey antibody to rabbit (fluorescein isothiocyanate) conjugate, 1:100; donkey antibody to mouse (Texas Red conjugate), 1:100 (Jackson Immunoresearch Laboratories). Cover slips were mounted with polyvinyl alcohol and 1,4-diazabicyclo[2.2.2]octane (Sigma) mounting medium and the slides were viewed under fluorescence with a Nikon Microphot-FXA microscope; the images were then scored and photographed.

23. A. Kumar, J. Haque, J. Lacoste, J. Hiscott, B. R. G. Williams, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6288 (1994); M. T. Diaz-Meco *et al.*, *Mol. Cell. Biol.* **13**, 4770 (1993); M. T. Diaz-Meco *et al.*, *EMBO J.* **13**, 1842 (1994); G. J. Schouten *et al.*, *ibid.* **16**, 3133 (1997).
24. J. A. DiDonato, M. Hayakawa, D. M. Rothwarf, E. Zandi, M. Karin, *Nature* **388**, 548 (1997); C. H. Regnier *et al.*, *Cell* **90**, 373 (1997).
25. M. Wilm and M. Mann, *Anal. Chem.* **68**, 1 (1996).

26. We thank I. Verma and R. Mueller for the gift of the IκB-α COOH-terminus construct and helpful discussions; J. DiDonato for the IκB-α vectors; H. Raymon and N. Richard for assistance with immunocytochemistry; S. Kim, A. LaPointe, and B. Lee for technical assistance; K. Davis and E. Carlson for help with compiling the manuscript and figures; and D. Anderson and A. Lewis for helpful comments and support. A.R. contributed to this work during a sabbatical at Signal Pharmaceuticals.

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IκB Kinase–β: NF-κB Activation and Complex Formation with IκB Kinase–α and NIK

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Activation of the transcription factor nuclear factor kappa B (NF-κB) by inflammatory cytokines requires the successive action of NF-κB–inducing kinase (NIK) and IκB kinase–α (IKK-α). A widely expressed protein kinase was identified that is 52 percent identical to IKK-α. IκB kinase–β (IKK-β) activated NF-κB when overexpressed and phosphorylated serine residues 32 and 36 of IκB-α and serines 19 and 23 of IκB-β. The activity of IKK-β was stimulated by tumor necrosis factor and interleukin-1 treatment. IKK-α and IKK-β formed heterodimers that interacted with NIK. Overexpression of a catalytically inactive form of IKK-β blocked cytokine-induced NF-κB activation. Thus, an active IκB kinase complex may require three distinct protein kinases.

Transcriptional activation of inflammatory response genes by tumor necrosis factor (TNF), interleukin-1 (IL-1), and other external stimuli is mediated by the transcription factor NF-κB (*1*, *2*). Normally, NF-κB is held in an inactive state in the cytoplasm by IκB inhibitory proteins. When cells are treated with TNF or IL-1, protein kinase cascades are activated that lead to phosphorylation of IκB proteins on two specific serine residues (*1*, *2*). This signal-induced phosphorylation targets IκB for ubiquitination and proteosome-mediated degradation, allowing nuclear translocation of NF-κB (*2*).

Several steps of the TNF- and IL-1–activated signaling pathways leading to IκB phosphorylation have now been elucidated (*3*–*10*). Both pathways merge at the level of the protein kinase NIK (NF-κB–inducing kinase) (*10*). The molecular mechanisms by which NIK becomes activated are not yet understood. However, the protein kinase CHUK is a downstream target of NIK (*11*) that directly associates with IκB-α and specifically phosphorylates it on serines 32 and 36 (*11*, *12*). These results have led to the redesignation of CHUK as IκB kinase–α (IKK-α).

IKK-α does not phosphorylate the two serines required for degradation of a second member of the IκB family, IκB-β, with equal efficiency; it has a marked preference for serine 23 over serine 19 (*11*). This finding indicates that another kinase might be responsible for IκB-β phosphorylation. Furthermore, mapping of the gene encoding CHUK revealed the presence of a CHUK-related sequence in the mouse genome (*13*). To search for IKK-α–related kinases, we screened the National Center for Biotechnology Information DNA database and identified an expressed sequence tag (EST) cDNA clone predicted to encode a polypeptide 57% identical to amino acids 624 to 658 of IKK-α (*14*). Full-length human cDNAs corresponding to the EST sequence were isolated from a Jurkat T cell cDNA library and found to encode a 756–amino acid protein very similar to IKK-α, which we designate IKK-β (Fig. 1). Overall, the sequences of IKK-α and IKK-β are 52% identical, with the NH_2-terminal kinase domains sharing 64% identity and the COOH-terminal regions, which contain leucine zipper and helix-loop-helix domains, having 44% sequence identity. An IKK-β mRNA of ~3.8 kb was detected by Northern (RNA) blot analysis in all tissues examined (*15*).

To determine whether IKK-β might have a role in NF-κB activation, we compared the ability of IKK-α and IKK-β to activate an NF-κB–dependent reporter gene in transiently transfected 293 cells (*16*). Overexpression of IKK-β gave consistently greater activation of the NF-κB reporter than did IKK-α at equivalent expression levels (Fig. 2A). A catalytically inactive mutant of IKK-β, IKK-$β_{(K44A)}$, failed to activate the NF-κB–dependent reporter gene when overexpressed and inhibited both TNF- and IL-1–induced NF-κB activation in a dose-dependent manner (Fig. 2B). In constrast, overexpression of wild-type IKK-β further enhanced TNF- and

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IL-1–induced NF-κB activation (*15*).

IKK-α directly phosphorylates IκB-α on serine residues 32 and 36, and this activity is enhanced by NIK stimulation (*12*). In similar in vitro phosphorylation assays (*11*), IKK-β was at least 20 times as active as IKK-α in phosphorylating bacterially produced IκB-α (Fig. 3A; compare lanes 1 and 3). IKK-β phosphorylated IκB-α on serines 32 and 36 with approximately equal efficiency (Fig. 3B). We also evaluated the ability of IKK-β to phosphorylate wild-type and mutant versions of the inhibitory protein IκB-β. IKK-β specifically phosphorylated IκB-β on its inducible phosphorylation sites, serine residues 19 and 23. IKK-β phosphorylated both sites equally well (Fig. 3B), in contrast to IKK-α, which preferentially phosphorylates serine 23 (*11*).

The activity of IKK-α is enhanced in cells that also overexpress NIK (*11*), but NIK-activated IKK-α is still somewhat less active than IKK-β expressed alone (*17*). NIK coexpression also enhanced IKK-β activity (Fig. 3A) but to a lesser extent than it activated IKK-α (*11*). When IKK-β was coexpressed with IKK-α (Fig. 3A, lane 7), or with both NIK and IKK-α (lane 8), there was no obvious increase in activity above that seen for IKK-β alone (lane 5).

The IκB-α–directed kinase activity of IKK-α is stimulated by TNF or IL-1 treatment (*12*). To determine whether the activity of IKK-β is also enhanced by inflammatory cytokines, we transiently expressed Flag-tagged IKK-β in HeLa cells. Thirty-six hours later, the cells were treated for 8 min with TNF or IL-1, and anti-Flag immunoprecipitates were examined for IκB kinase activity. Both TNF and IL-1 treatment resulted in increased phosphorylation of exogenous IκB-α (Fig. 3C), demonstrating that IKK-β is a cytokine-activated IκB kinase.

Because IKK-α directly interacts with both NIK and IκB-α (*11*), we examined whether IKK-β also associates with these two proteins in cotransfection, coimmunoprecipitation experiments (*18*). NIK associated with IKK-β, although this interaction was weaker than NIK's interaction with IKK-α (Fig. 4A). To measure association with IκB-α, Flag–IKK-β was expressed with wild-type or mutant IκB-α in 293 cells, and anti-Flag immunoprecipitates were analyzed for coprecipitating IκB-α by immunoblotting. No interaction was detected between wild-type IKK-β and IκB-α when these two proteins were coexpressed. However, IKK-β interacted weakly with an IκB-α mutant in which serines 32 and 36 were replaced by alanine (Fig. 4B). We also performed these experiments in the presence of coexpressed p65 and p50 subunits of NF-κB, which stabilize overexpressed IκB-α (*11*, *19*) (Fig. 4B). This made it possible to detect the interaction of IKK-β with IκB-α and improved the interaction of IKK-β with IκB-$\alpha_{(S32,36A)}$ (Fig. 4B). Even stronger interactions were observed between the catalytically inactive IKK-$\beta_{(K44A)}$ mutant and IκB-α. Overall, the behavior of IKK-β in these assays is similar to that of IKK-α, whose affinity for IκB-α is reduced after phosphorylation of IκB-α on serines 32 and 36 (*11*). Coexpression of IKK-β and IκB-α resulted in the detection of a more slowly migrating, phosphorylated IκB-α species in immunoblot analysis of total cell extracts (Fig. 4B). In similar experiments performed with IKK-α, this was only observed if NIK was also expressed (*11*).

The COOH-terminal regions of both IKK-α and IKK-β contain putative helix-loop-helix and leucine zipper motifs (*20*) (Fig. 1). Because such domains often participate in protein oligomerization, we examined whether these two kinases could form dimers. Ex-

Fig. 1. Sequence alignment of human IKK-β and IKK-α (*23*). An optimized alignment of the protein sequences of human IKK-β and IKK-α is shown. Identical amino acids are boxed. Brackets delineate the boundaries of the kinase domains. Asterisks identify a putative leucine zipper region, and two amphipathic helices of a helix-loop-helix domain are underlined. The IKK-β nucleotide sequence has been deposited in GenBank (accession number AF029684).

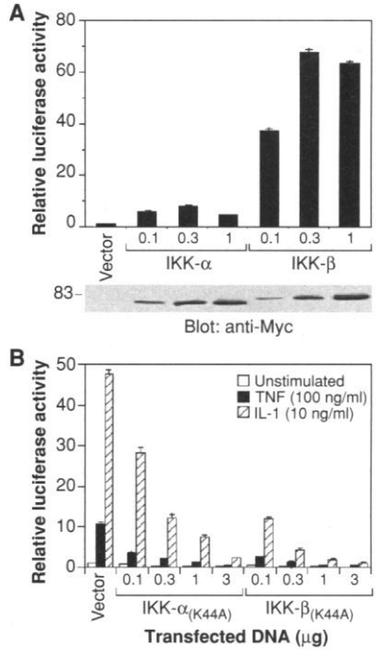

Fig. 2. Comparison of IKK-α and IKK-β in NF-κB activation. (**A**) Effect of IKK-α and IKK-β overexpression on NF-κB–dependent reporter gene activity in 293 cells. Two hundred ninety-three cells were transiently cotransfected (*16*) with an E-selectin-luciferase reporter gene plasmid and expression vectors encoding Myc-tagged IKK-α or IKK-β, or with a vector control. Luciferase activities were determined and normalized on the basis of β-galactosidase (β-Gal) expression from cotransfected pRSV–β-Gal (1 mg). The values shown are averages (mean ± SEM) of one representative experiment (out of five) in which each transfection was performed in duplicate. The amounts of IKK-α and IKK-β expressed in each sample were determined by immunoblotting with polyclonal antibodies to Myc (anti-Myc; lower panel). (**B**) Effect of IKK-$\alpha_{(K44A)}$ and IKK-$\beta_{(K44A)}$ expression on TNF- and IL-1–induced reporter gene activity in 293 cells; 293 and 293/IL-1R1 cells (*8*) were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and a vector control or with IKK-$\alpha_{(K44A)}$ or IKK-$\beta_{(K44A)}$ expression vectors as indicated. Thirty to 36 hours after transfection, cells were either left untreated (open bars) or were stimulated for 6 hours with TNF (100 ng/ml) (solid bars) or IL-1 (10 ng/ml) (hatched bars); 293 cells were stimulated with TNF, and 293/IL-1R1 cells were stimulated with IL-1. Luciferase activities were determined and normalized on the basis of β-Gal expression. Values shown are the averages (mean ± SEM) of duplicate samples from one representative experiment.

pression vectors encoding various combinations of Flag- and Myc-epitope–tagged proteins were transfected into 293 cells, and coimmunoprecipitation analyses were done (*18*). Both homo- and heterodimers were detected, with the α/β heterodimer and the α/α homodimer forming more readily than the β/β homodimer (Fig. 5A). When Flag-tagged IKK-α was coexpressed with both Myc–IKK-α and Myc–IKK-β, anti-Flag immunoprecipitates preferentially contained Myc–IKK-β. Likewise, Flag–IKK-β bound more readily to Myc–IKK-α than to Myc–IKK-β (Fig. 5A). Thus, IKK-α and IKK-β may naturally exist in a heterodimeric state.

We raised antibodies to peptides from the two kinases and tested for association of the endogenous kinases in untransfected 293 cells (*21*). Protein immunoblot analysis of IKK-β immunoprecipitates showed the presence of IKK-α, demonstrating that heterodimers of IKK-α and IKK-β exist under physiological conditions (Fig. 5B). This would suggest that the 85- and 87-kD proteins purified by DiDonato *et al.* (*12*) in roughly equivalent amounts may correspond to IKK-α and IKK-β, respectively.

The existence of IKK homo- and heterodimers and the ability of NIK to interact with both IKK isoforms suggested two possible types of NIK-IKK complexes. In one scenario, monomeric IKK might interact with either another IKK subunit or with NIK. Alternatively, NIK might associate with dimeric forms of IKK, and thus all three kinases could exist in a single complex. To address these

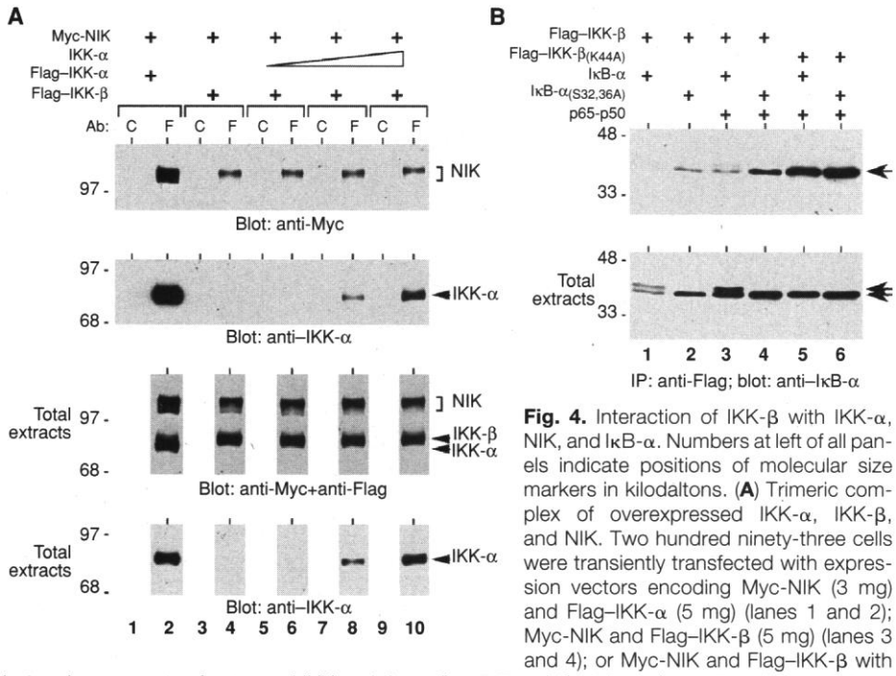

Fig. 4. Interaction of IKK-β with IKK-α, NIK, and IκB-α. Numbers at left of all panels indicate positions of molecular size markers in kilodaltons. (**A**) Trimeric complex of overexpressed IKK-α, IKK-β, and NIK. Two hundred ninety-three cells were transiently transfected with expression vectors encoding Myc-NIK (3 mg) and Flag–IKK-α (5 mg) (lanes 1 and 2); Myc-NIK and Flag–IKK-β (5 mg) (lanes 3 and 4); or Myc-NIK and Flag–IKK-β with increasing amounts of untagged IKK-α: 0.3 mg (lanes 5 and 6), 1.0 mg (lanes 7 and 8), or 3.0 mg (lanes 9 and 10). After 36 hours, cell lysates were immunoprecipitated with monoclonal anti-Flag (lanes labeled F) or control mouse IgG (lanes labeled C). Coprecipitating Myc-NIK proteins were detected with anti-Myc (top panel), and coprecipitating IKK-α proteins were detected with anti–IKK-α (second panel). Portions of total cell extracts were also immunoblotted with anti-Myc and anti-Flag (third panel) or anti–IKK-α (bottom panel). (**B**) Coprecipitation of overexpressed IKK-β and IκB-α; 293 cells (2×10^6) were transiently transfected with equivalent amounts (3 mg) of the indicated expression plasmids. After 30 hours, Flag–IKK-β proteins were immunoprecipitated (IP) with monoclonal anti-Flag. Coprecipitating IκB-α was detected by immunoblot analysis (upper panel). Portions of total cell extracts (10 ml) of the same transfections were also immunoblotted with polyclonal anti-IκB-α (lower panel). Arrows mark the positions of unphosphorylated IκB-α proteins and of IκB-α phosphorylated on serines 32 and 36.

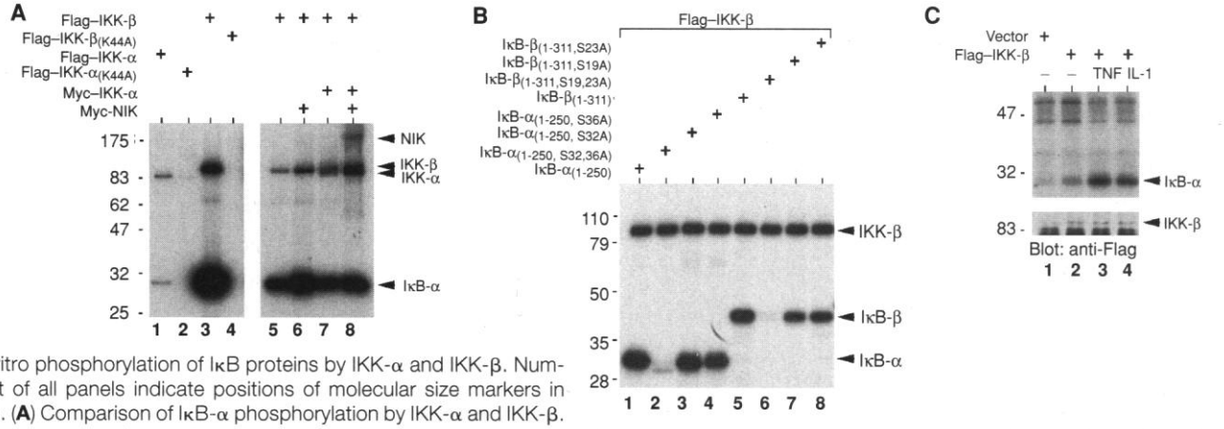

Fig. 3. In vitro phosphorylation of IκB proteins by IKK-α and IKK-β. Numbers at left of all panels indicate positions of molecular size markers in kilodaltons. (**A**) Comparison of IκB-α phosphorylation by IKK-α and IKK-β. 293 cells were transiently transfected with the indicated epitope-tagged expression vectors. Twenty-four hours after transfection, IKK proteins were immunoprecipitated with monoclonal antibody to Flag (anti-Flag) affinity resin and purified as described (*24*). Purified IKK proteins as indicated (lanes 1 through 5) or purified IKK-β with coprecipitated NIK (lane 6), IKK-α (lane 7), or IKK-α and NIK (lane 8), were used in in vitro kinase reactions with bacterially expressed IκB-$\alpha_{(1\text{-}250)}$ and [γ-^{32}P]ATP. Arrowheads indicate the positions of IKK proteins, NIK, and IκB-$\alpha_{(1\text{-}250)}$ proteins. Lanes 3 and 5 contain identical samples; the two halves of the figure were exposed for different lengths of time. (**B**) Specificity of IκB phosphorylation by IKK-β. Flag–IKK-β protein, purified as described above (*24*), was used in in vitro kinase reactions with bacterially expressed IκB-$\alpha_{(1\text{-}250)}$ proteins with the indicated mutations (lanes 1 through 4) or the indicated IκB-$\beta_{(1\text{-}311)}$ proteins (lanes 5 through 8). Arrowheads indicate the positions of IKK-β, IκB-$\beta_{(1\text{-}311)}$, and IκB-$\alpha_{(1\text{-}250)}$. (**C**) Cytokine activation of IKK-β in mammalian cells. HeLa cells were transiently transfected with vector control or a Flag–IKK-β expression plasmid. Thirty-six hours after transfection, the cells were treated with TNF (100 ng/ml) or IL-1 (10 ng/ml) for 8 min, and the IKK-β protein was immunoprecipitated with monoclonal anti-Flag. The immunoprecipitates were used in in vitro kinase reactions with bacterially expressed IκB-$\alpha_{(1\text{-}250)}$ and [γ-^{32}P]ATP. As a control for IKK-β protein expression, one-half of the in vitro kinase reactions were analyzed by immunoblotting with polyclonal anti-Flag (lower panel). Arrowheads indicate the positions of IκB-$\alpha_{(1\text{-}250)}$ and IKK-β proteins.

two alternatives, we coexpressed Flag-tagged IKK-β and Myc-tagged NIK with increasing amounts of untagged IKK-α. IKK-β was immunoprecipitated from extracts with antibodies to Flag, and coprecipitating NIK and IKK-α were detected by immunoblotting (Fig. 4A). Increased amounts of IKK-α resulted in conversion of IKK-β homodimers to the preferred IKK-α/IKK-β heterodimeric state (see Fig. 5A), and NIK was still precipitated (Fig. 4A). Thus, NIK appears to interact with an IKK-α/IKK-β heterodimer.

The fact that NIK, IKK-α, and IKK-β can exist in a ternary complex suggests that the IKKs have distinct or multiple domains for protein-protein interaction. We tested a series of IKK-β deletion mutants for interaction with IKK-α, IKK-β, NIK, and IκB-α (Fig. 6). The region of IKK-β containing the leucine zipper–like motif was required both for homodimerization of IKK-β and for formation of heterodimers with IKK-α, whereas the putative helix-loop-helix region was dispensible. Mutant IKK-β proteins consisting of the kinase domain alone or lacking this domain entirely both associated with NIK, indicating that multiple regions of IKK-β can bind to NIK (Fig. 6). Interaction of IKK-β with its substrate IκB-α required both the kinase and leucine zipper domains, suggesting that IKK-β dimerization may be necessary for recognition of IκB-α.

The results described here identify IKK-β as a second cytokine-activated IκB kinase. Compared to IKK-α, IKK-β is more active in NF-κB reporter gene assays and as an IκB kinase. Likewise, IKK-α and IKK-β have somewhat different substrate specificities. IKK-β phosphorylates the α and β members of the IκB family of inhibitory proteins on the appropriate serine residues, whereas IKK-α phosphorylates serine 19 of IκB-β quite poorly. IKK-α and IKK-β may normally exist as a leucine zipper–linked heterodimer that can interact directly with the upstream kinase NIK. Furthermore, catalytically inactive versions of NIK (*10*), IKK-α (*11*), and IKK-β independently block the activation of NF-κB triggered by either TNF or IL-1. One interpretation of these results is that all three kinases are components of the large 700- to 900-kD IκB kinase complex (*12*, *22*), and each is essential for activity of the complex. IκB kinase complexes containing homodimers of IKK-α and IKK-β may also exist, each having somewhat different properties and therefore providing variations on the common theme of signal-regulated IκB phosphorylation.

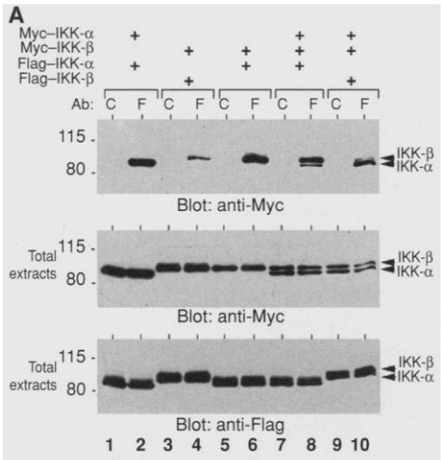

Fig. 5. Interaction of IKK-β with IKK-α. Numbers at left of panels indicate positions of molecular size markers in kilodaltons. (**A**) Two hundred ninety-three cells (2×10^6) were transiently transfected with equivalent amounts (3 mg) of the indicated expression plasmids. After 36 hours, cell lysates were immunoprecipitated with monoclonal anti-Flag (lanes labeled F) or control mouse IgG (lanes labeled C). Coprecipitating Myc-IKK proteins were detected by immunoblot with polyclonal anti-Myc (upper panel). Portions of total cell extracts (10 ml) were also immunoblotted with anti-Myc (middle panel) or with polyclonal anti-Flag (bottom panel). Arrowheads indicate the positions of IKK-α and IKK-β proteins. (**B**) IKK-α/β heterodimers. Coimmunoprecipitation was done with polyclonal anti–IKK-α and anti–IKK-β (*21*). Two hundred ninety-three cell lysates were incubated with anti–IKK-β, and coprecipitating IKK-α was detected by immunoblot analysis (*21*). Ab, antibody; Pre, pre-immune serum.

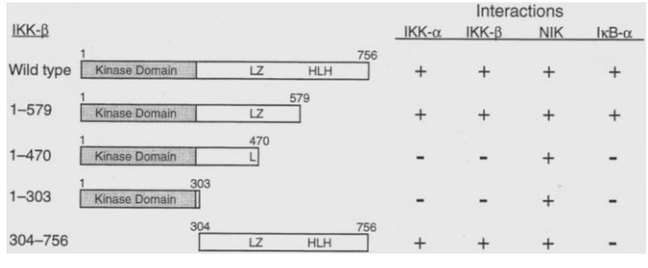

Fig. 6. Analysis of IKK-β deletion mutants. The horizontal bars represent the sequence of IKK-β, with kinase (shaded), leucine zipper (LZ), and helix-loop-helix (HLH) domains indicated. The amino acids contained in each deletion mutant are indicated. Interactions of the Myc–IKK-β mutants with Flag–IKK-α, Flag–IKK-β, Flag-NIK, and Flag–IκB-α were determined by coimmunoprecipitation assays of 293 cells (*18*). A plus sign indicates that the two proteins did associate upon overexpression, whereas a minus sign indicates that no association was detected. The expression of all proteins was confirmed by immunoblot analysis of total cell lysates. Results are representative of two independent experiments.

REFERENCES AND NOTES

1. P. A. Baeuerle and T. Henkel, *Annu. Rev. Immunol.* **12**, 141 (1994); I. M. Verma, J. K. Stevenson, E. M. Schwartz, D. Van Antwerp, S. Miyamoto, *Genes Dev.* **9**, 2723 (1995); P. A. Baeuerle and D. Baltimore, *Cell* **87**, 13 (1996).
2. D. Thanos and T. Maniatis, *Cell* **80**, 529 (1995).
3. H. Hsu, J. Xiong, D. V. Goeddel, *ibid.* **81**, 495 (1995).
4. H. Hsu, H. B. Shu, M. P. Pan, D. V. Goeddel, *ibid.* **84**, 299 (1996).
5. H. Hsu, J. Huang, H. B. Shu, V. Baichwal, D. V. Goeddel, *Immunity* **4**, 387 (1996).
6. H. B. Shu, M. Takeuchi, D. V. Goeddel, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13973 (1996).
7. J. Huang, X. Gao, S. Li, Z. Cao, *ibid.*, in press.
8. Z. Cao, W. J. Henzel, X. Gao, *Science* **271**, 1128 (1996).
9. Z. Cao, J. Xiong, M. Takeuchi, T. Kurama, D. V. Goeddel, *Nature* **383**, 443 (1996).
10. N. L. Malinin, M. P. Boldin, A. V. Kovalenko, D. Wallach, *ibid.* **385**, 540 (1997); H. Y. Song *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9792 (1997).
11. C. H. Régnier *et al.*, *Cell* **90**, 373 (1997).
12. J. A. DiDonato, M. Hayakawa, D. M. Rothwarf, E. Zandl, M. Karin, *Nature* **388**, 548 (1997).
13. B. A. Mock, M. A. Connelly, O. W. McBride, C. A. Kozak, K. B. Marcu, *Genomics* **27**, 348 (1995).
14. GenBank accession number W68756.
15. J. Woronicz and D. Goeddel, unpublished data.
16. Mammalian cell expression vectors encoding IKK-β and IKK-$\beta_{(K44A)}$ were constructed in the previously described pRK5 plasmid [T. J. Schall *et al.*, *Cell* **61**, 361 (1990)]. The IKK-$\beta_{(K44A)}$ mutant was generated by polymerase chain reaction (PCR). The expression plasmids for IKK-α and IKK-$\alpha_{(K44A)}$ are described in (*11*). Transient transfection of 293 cells with the use of the calcium phosphate precipitation method was done as described in (*11*).
17. X. Gao and Z. Cao, unpublished data.
18. The coimmunoprecipitation experiments were done as described in (*11*). The NH_2-terminal Myc–IKK-β and Myc–IKK-β deletion mutants (see Fig. 6) were generated in pRK5 by means of PCR.
19. M. L. Scott, T. Fujita, H. C. Liou, G. P. Nolan, D. Baltimore, *Genes Dev.* **7**, 1266 (1993).
20. M. A. Connelly and K. B. Marcu, *Cell. Mol. Biol. Res.* **41**, 537 (1995).
21. Polyclonal rabbit antisera to IKK-α and IKK-β were raised by BAbCO (Richmond, CA) against peptides corresponding to amino acids 672 to 700 of IKK-α and amino acids 467 to 500 of IKK-β. For coprecipitation of endogenous proteins, lysates from 293 cells (4×10^7) (*11*) were incubated for several hours with 1 ml of antiserum to IKK-β or preimmune serum and 20 ml of protein A–agarose beads (Oncogene Science). Coprecipitating IKK-α was detected by immunoblot analysis (*11*) with antibodies to IKK-α with the use of the Enhanced Chemiluminscence (ECL) Plus Western Blotting Detection System (Amersham).
22. Z. J. Chen, L. Parent, T. Maniatis, *Cell* **84**, 853 (1996).
23. 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.
24. The in vitro phosphorylation assays were done as described in (*11*). The COOH-terminal Flag–IKK-β and IKK-$\beta_{(K44A)}$ were constructed in-frame with DNA encoding the Flag epitope in pRK5. The Myc- and Flag–IKK-α, Myc-NIK, IκB-$\alpha_{(1\text{-}250)}$, and IκB-$\beta_{(1\text{-}311)}$ wild-type and mutant expression plasmids are described in (*11*). The amounts of purified Flag-IKK proteins used in the kinase reactions were determined by immunoblotting with polyclonal antibodies to Flag (Santa Cruz Biotechnology). Only experiments with comparable amounts of Flag-IKK proteins were taken into consideration.

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