

this family of domains mediates a host of biological processes, including protein internalization and signaling.

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16. A DNA fragment encoding amino acids 121 through 218 of human Eps15 followed by a TAA stop codon was cloned into the Bam HI and Xho I sites of the pRSETA vector (Clontech). Trp¹⁶⁹ → Ala¹⁶⁹ and Trp¹⁶⁹ → Tyr¹⁶⁹ mutations were created by site-directed mutagenesis with the use of the Quick-change system (Stratagene) and were verified by dideoxysequencing. Wild-type EH₂ and mutants were transformed into *Escherichia coli* strains BL21 pLys S and B834 pLys S. Unlabeled or uniformly isotope labeled protein was obtained with LB broth or M9-minimal media containing ¹⁵NH₄Cl and ¹³C₆-glucose. His₆-tagged EH₂ was purified with Talon resin (Clontech) and cleaved with Enterokinase (Novagen), followed by protease removal with EKapture agarose (Novagen). A matrix-assisted laser desorption/ionization (MALDI) experiment of imidazole-eluted His₆-tagged EH₂ confirmed the size of 15.45 kD. Amino-terminal sequencing indicated that about 50% of EH₂ lacks the first two amino acids (encoded by the vector), which does not influence the folded structure. EH₂ is monomeric as determined by equilibrium sedimentation ultracentrifugation and pulsed field gradient diffusion experiments [A. S. Altieri, D. P. Hinton, R. A. Byrd, *J. Am. Chem. Soc.* **117**, 7566 (1995)]. All NMR spectra were recorded on samples containing 1 to 4 mM EH₂, 20 mM perdeuterated tris, 0.1 M KCl, 2 mM CaCl₂, 0.1 mM perdeuterated di-thiothreitol, 10 μM 4-amidinophenylmethane sulfonyl fluoride, and 2 mM NaNa₂ (pH = 7.0) dissolved in either 5 or 99.99% ²H₂O/H₂O. Calcium binding was assessed from NMR spectra of EH₂ in the presence of calcium and after addition of 10 mM EDTA. Even after repeated EDTA treatment at 37°C, approximately 20% of EH₂ still contained calcium (Fig. 3A). NMR experiments were recorded at 25°C on Varian INOVA 500-MHz and 600-MHz spectrometers equipped with shielded triple resonance probes. NMR experiments [D. R. Muhandiram and L. E. Kay, *J. Magn. Reson. B* **103**, 203 (1994); L. E. Kay, *Biochem. Cell. Biol.* **75**, 1 (1997)] were recorded essentially as described [M. Overduin, K. I. Tong, C. M. Kay, M. Ikura, *J. Biomol. NMR* **7**, 173 (1996)]. Spectra were analyzed with NMRPipe [F. Delaglio et al., *ibid.* **6**, 277 (1995)] and in-house software on Sun Microsystems and Silicon Graphics workstations. Interproton distance restraints (313 intraresidue, 491 sequential, 509 short-range, and 432 long-range) were derived from

three-dimensional (3D) and 4D isotope-filtered NOE spectra and were treated as described [C. M. Fletcher, D. N. M. Jones, R. Diamond, D. Neuhaus, *ibid.* **8**, 292 (1996)]. Dihedral restraints including 59 ϕ angles and 45 ψ angles were included based on ³J_{HN-Hα} coupling constants and on ¹H_α, ¹³C_α, and ¹³C' chemical shifts [D. S. Wishart and B. D. Sykes, *ibid.* **4**, 171 (1994)]. Slowly exchanging amide protons were identified from several ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra recorded over a week, after lyophilized protein was dissolved in 99.99% ²H₂O. In the final calculations, 20 pairs of hydrogen bond restraints were included and six distance restraints between calcium and coordinating oxygen atoms were added, based on average distances in EF hand proteins (19). Fifty structures were calculated with the r⁻⁶ summation method in a restrained molecular dynamics simulated annealing protocol within X-PLOR 3.84 [A. Brünger, *X-PLOR*, version 3.7 (Yale Univ. Press, New Haven, CT, 1992)]. Distance and dihedral angle restraints were treated with soft-square-well energy potentials and force constants of 50 kcal mol⁻¹ Å². The total, distance violation, and dihedral violation energies were 307 ± 12 kcal mol⁻¹, 57.0 ± 4.5 kcal mol⁻¹, and 0.68 ± 0.6 kcal mol⁻¹, respectively. No distance and angle restraints were violated by more than 0.35 Å and 2°, respectively. The rmsds from idealized geometry for bonds, angles, and impropers were 0.0028 ± 0.0001 Å, 0.62 ± 0.012°, and 0.47 ± 0.076°, respectively. Ramachandran plot analysis of the 20 structures with Procheck-NMR (20) showed that 85.8, 11.3, 2.5, and 0.5% of the non-Gly and non-Pro residues were in the most favorable, additional allowed, generously allowed, and disallowed regions, respectively.

17. 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.

18. Approximately 68 resonance units (RU) of NH₂-terminal biotinylated PTGSSTNPFL (17) peptide (Research Genetics) were attached to a streptavidin-coated sensor chip in a BIAcore 2000 instrument. Wild-type EH₂ and the two Trp¹⁶⁹ mutants were injected over the peptide in eight concentrations ranging from 2 μM to 1.2 mM, with a flow rate of 30 μL min⁻¹ in 20 mM Pipes (pH 7.3), 50 mM KCl, 1 mM

β-mercaptoethanol, 2 mM CaCl₂, 100 μM sodium azide, and 0.005% Tween-20. Equilibrium RU values were estimated at each protein concentration by averaging the steady-state response after injection and correcting for the sample refractive index component. The K_D was estimated from the relation between the EH₂ concentration and equilibrium RU, using steady-state affinity analysis (BIAevaluation 2.1).

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21. Superposition of residues 121 through 215 yields rmsds of 0.54 ± 0.08 Å and 0.92 ± 0.10 Å for the backbone and all heavy atoms, respectively.

22. The disordered five NH₂-terminal residues [DRWGS (17)], which are encoded by the expression vector, and the six COOH-terminal residues, which are derived from the third EH domain (KTW) and expression vector (ELI), are not depicted.

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24. We thank R. Muhandiram and L. E. Kay for NMR pulse sequences, J. Mamay for computational support, S. A. Johnson for assistance with BIAcore experiments, P. P. Di Fiore for cDNA, B. Thimmig and C. McHenry for assistance with sedimentation equilibrium experiments, K. Clay and R. Murphy for MALDI data, and J. Enmon, D. Jones, and F. Tebar for insightful discussions. The NMR Center is supported by the Howard Hughes Medical Institute (HHMI). The University of Colorado Cancer Center Facilities for DNA and Protein Sequencing are supported by NIH. This research is funded by NIH and the HHMI (A.S. and M.O.) and by the American Cancer Society and Pew Scholar's Program (M.O.). T.B. and R.E.C. are recipients of NIH and Cancer League of Colorado postdoctoral fellowships, respectively. The coordinates have been deposited in the Brookhaven Protein Data Bank under accession number 1eh2.

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Direct Phosphorylation of IκB by IKKα and IKKβ: Discrimination Between Free and NF-κB-Bound Substrate

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A large protein complex mediates the phosphorylation of the inhibitor of κB (IκB), which results in the activation of nuclear factor κB (NF-κB). Two subunits of this complex, IκB kinase α (IKKα) and IκB kinase β (IKKβ), are required for NF-κB activation. Purified recombinant IKKα and IKKβ expressed in insect cells were used to demonstrate that each protein can directly phosphorylate IκB proteins. IKKα and IKKβ were found to form both homodimers and heterodimers. Both IKKα and IKKβ phosphorylated IκB bound to NF-κB more efficiently than they phosphorylated free IκB. This result explains how free IκB can accumulate in cells in which IKK is still active and thus can contribute to the termination of NF-κB activation.

The IKK complex, isolated from extracts of HeLa cells treated with the proinflammatory cytokine TNF (tumor necrosis factor), phosphorylates two regulatory serine residues at

the NH₂-termini of the NF-κB inhibitors IκBα and IκBβ (1). This phosphorylation event triggers the polyubiquitination of IκBs followed by their degradation through the

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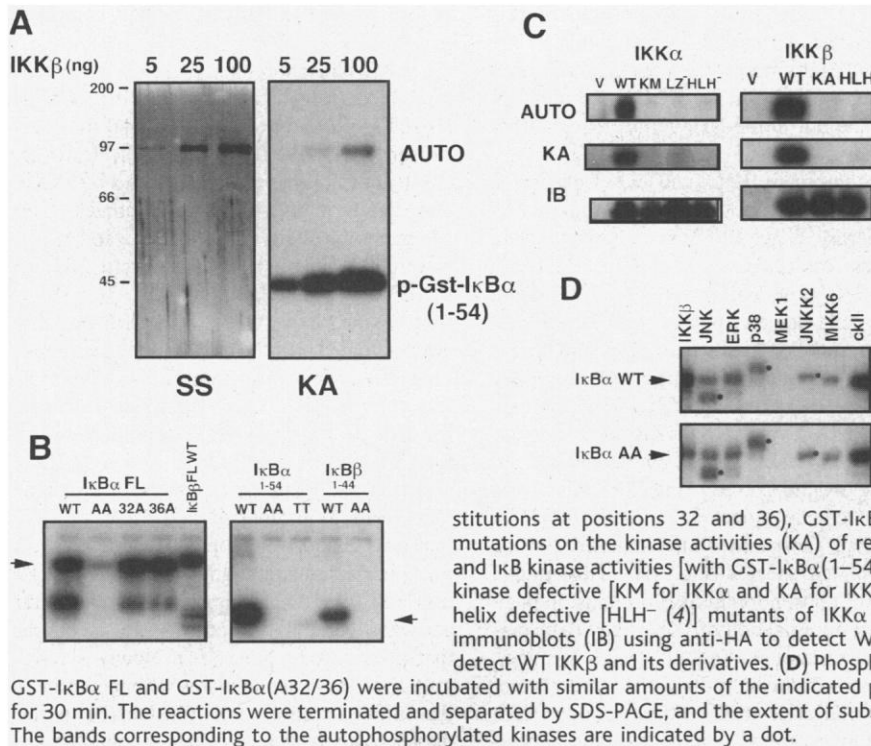


Fig. 1. Direct phosphorylation of I κ B by IKK α and IKK β . (A) I κ B α phosphorylation by purified recombinant IKK β . The protein was expressed using a baculovirus vector in Sf9 (9) cells and purified as described (10). The indicated amounts of highly purified IKK β were separated by SDS-PAGE and visualized by silver staining (SS). These were also examined for phosphorylation of GST-I κ B α (1–54) using a standard I κ B kinase assay (KA). The phosphorylated GST-I κ B(1–54) and IKK β (Auto) bands are indicated. The migration positions of molecular size markers (in kilodaltons) are also indicated. Similar results were obtained for recombinant IKK α (11). (B) Substrate specificity of purified recombinant IKK β was examined using GST-I κ B α FL (full length) wild-type (WT), GST-I κ B α FL with Ala at positions 32 and 36 (AA) or with single Ala substitutions at positions 32 (32A) or 36 (36A), GST-I κ B β FL, GST-I κ B α (1–54) WT, GST-I κ B α (1–54) AA, GST-I κ B α (1–54) TT (Thr substitutions at positions 32 and 36), GST-I κ B β (1–44) WT, and GST-I κ B β (1–44) AA. (C) Effect of mutations on the kinase activities (KA) of recombinant IKK α and IKK β . Autophosphorylation (Auto) and I κ B kinase activities [with GST-I κ B α (1–54) as a substrate] of purified recombinant wild type (WT), kinase defective [KM for IKK α and KA for IKK β (4)], leucine zipper defective [LZ⁻ (4)], and helix-loop-helix defective [HLH⁻ (4)] mutants of IKK α and IKK β , were determined. The bottom panels show immunoblots (IB) using anti-HA to detect WT IKK α and its derivatives (left) and anti-FLAG (M2) to detect WT IKK β and its derivatives. (D) Phosphorylation of I κ B α by different protein kinases. Wild-type I κ B α WT and I κ B α AA were incubated with similar amounts of the indicated protein kinases in standard kinase buffer and [γ -³²P]ATP for 30 min. The reactions were terminated and separated by SDS-PAGE, and the extent of substrate phosphorylation was revealed by autoradiography. The bands corresponding to the autophosphorylated kinases are indicated by a dot.

26S proteasome, and thereby leads to NF- κ B activation (2). IKK is a large, 900-kD, protein complex that is composed of multiple subunits. Two of these subunits, IKK α and IKK β , are serine kinases (1, 3–6). Epitope-tagged IKK α and IKK β , when produced by cell-free translation in reticulocyte lysates or by transient transfection of mammalian cells, are incorporated into the IKK complex, which can be isolated by immunoprecipitation of either IKK α or IKK β (1, 4). The I κ B kinase activity of the entire complex is rapidly stimulated by TNF or interleukin 1 (IL-1), with kinetics matching those of I κ B α phosphorylation and degradation (1, 4).

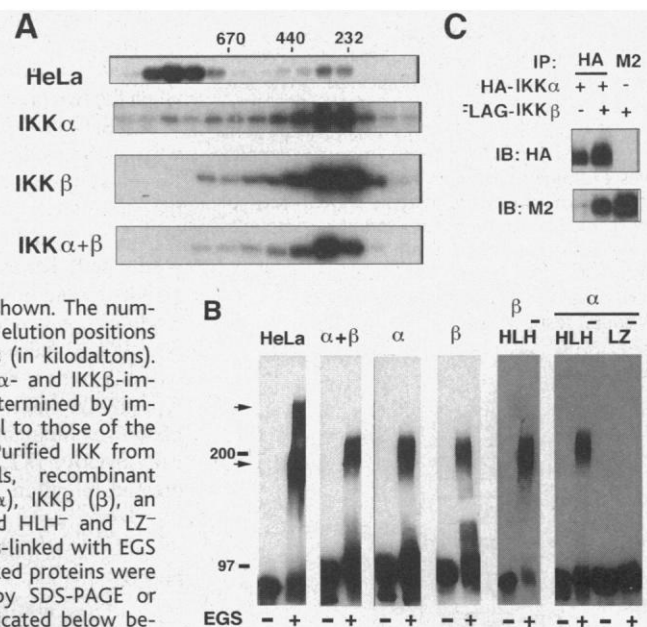
Because expression and immunoprecipitation of catalytically inactive IKK α or IKK β mutants still resulted in isolation of a cytokine-inducible I κ B kinase activity (4), the question was raised as to whether IKK α and IKK β are directly responsible for I κ B phosphorylation (7). Indeed, no I κ B kinase activity was obtained after translation of IKK α or IKK β in wheat germ extracts or after expression in *Escherichia coli* (1, 4, 8). Although transient expression of catalytically inactive IKK α or IKK β mutants inhibits NF- κ B activation (1, 3–6), these results are entirely compatible with IKK α and IKK β being upstream kinases that activate another subunit

of the IKK complex, which in turn phosphorylates I κ B (7).

We used a baculovirus expression system to express (His)₆-HA-tagged IKK α and

(His)₆-FLAG-tagged IKK β separately or together in Sf9 insect cells (9). Recombinant IKK α and IKK β were purified to apparent homogeneity from baculovirus-infected in-

Fig. 2. Formation of dimers by IKK α and IKK β . (A) The Superose 6 elution profile of the purified IKK complex from TNF- α -treated HeLa cells was compared to the elution profiles of purified recombinant IKK α , IKK β , and IKK α -IKK β (α + β) expressed in Sf9 cells (1, 4). The kinase activities toward GST-I κ B α (1–54) are shown. The numbers at the top indicate the elution positions of molecular size standards (in kilodaltons). The elution profiles of IKK α - and IKK β -immunoreactive materials determined by immunoblotting were identical to those of the kinase activities (11). (B) Purified IKK from TNF- α -treated HeLa cells, recombinant IKK α -IKK β (α + β), IKK α (α), IKK β (β), an HLH⁻ mutant of IKK β , and HLH⁻ and LZ⁻ mutants of IKK α were cross-linked with EGS as described (13). Cross-linked proteins were either directly separated by SDS-PAGE or immunoprecipitated as indicated below before separation by SDS-PAGE. In either case, proteins were detected by immunoblotting. For the IKK complex from HeLa cells (HeLa), a representative blot shows an immunoblot (with anti-IKK α) of proteins that were immunoprecipitated with anti-IKK β . An identical pattern of bands was observed when the blot was stripped and reprobed with anti-IKK β (13). For the (HA)-IKK α -(FLAG)-IKK β complex (α + β), the cross-linked proteins were immunoprecipitated with anti-HA and immunoblotted with anti-FLAG (M2). For (HA)-IKK α (α), (FLAG)-IKK β (β), and the various mutants, the cross-linked proteins were directly resolved by SDS-PAGE and probed with anti-HA for IKK α and anti-FLAG (M2) for IKK β . (C) Association of (HA)-IKK α and (FLAG)-IKK β in coinfecting Sf9 cells. (HA)-IKK α and (FLAG)-IKK β were expressed either separately or together as indicated. The antibodies used for immunoprecipitation (IP) and immunoblotting (IB) were anti-HA and anti-FLAG (M2), as indicated.



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sect cells (10). These purified proteins (Fig. 1A) (11) phosphorylated I κ B α specifically on Ser³² and Ser³⁶ and I κ B β on Ser¹⁹ and Ser²³ (Fig. 1B) (11). Mutants of the NH₂-terminal regulatory domains of both I κ Bs in which threonine was substituted for these serine residues were not phosphorylated (Fig. 1B) (11). Mutants of IKK α and IKK β that are defective in generation of IKK activity in mammalian cells (4) were also expressed in Sf9 cells and purified. These mutants showed little or no I κ B kinase activity (Fig. 1C). Hence, IKK α and IKK β appear to be direct I κ B kinases. Comparison of purified IKK β to a panel of seven other kinases indicated that it was the only kinase that specifically phosphorylated I κ B α at Ser³² and Ser³⁶, as revealed by the differences in ³²P incorporation into wild-type I κ B α and the I κ B α (A32/36) mu-

tant (Fig. 1D). Even though some protein kinases such as casein kinase II (CKII) are efficient I κ B kinases, the sites they phosphorylate are located in the COOH-terminal domain and not in the NH₂-terminal regulatory domain (12).

Recombinant IKK α and IKK β expressed alone or together (IKK α -IKK β) migrated on a Superose 6 gel filtration column with an apparent molecular size of 230 to 250 kD (Fig. 2A). The native IKK complex from HeLa cells has an apparent size of 900 kD (7). Thus, the Sf9 cells may lack components that are present in the large IKK complex. Chemical cross-linking (13) of recombinant IKK α , IKK β , or IKK α -IKK β complexes resulted in appearance of a single species migrating around 200 kD (Fig. 2B). Mutations in the leucine zipper (LZ) motif of IKK α abolished formation of the dimeric 200-kD cross-linked species (Fig. 2B). This mutant does not phosphorylate I κ B proteins, indicating that dimerization of IKK α may be necessary for formation of a functional I κ B kinase. Mutations in the helix-loop-helix (HLH) motifs of IKK α or IKK β did not abolish their homodimerization, as judged by the appearance of the 200-kD cross-linked species (Fig. 2B). However, these mutations did result in loss of kinase activity (Fig. 1C). Thus, the HLH motifs of IKK α and IKK β are necessary for catalytic activity even in the absence of other regulatory proteins.

Immunoprecipitation experiments with HA-tagged IKK α and FLAG-tagged IKK β revealed formation of stable heterodimers when the two proteins were expressed together in Sf9 cells (Fig. 2C). We examined whether heterodimers in which one subunit was catalytically inactive would still catalyze the phosphorylation of I κ B α (Fig. 3). Complexes of catalytically inactive IKK α and wild-type IKK β exhibited similar I κ B kinase activity to that of wild-type IKK α -IKK β heterodimers. A heterodimer composed of wild-type IKK α and catalytically inactive IKK β was also active, albeit not as active as wild-type IKK α homodimer. These results explain why immunoprecipitation of catalytically inactive IKK α or IKK β transiently expressed in cultured mammalian cells results

in formation of partially active IKK complexes (4).

IKK β phosphorylated a fusion protein of glutathione transferase with the first 54 amino acids of I κ B [GST-I κ B α (1-54)] more efficiently than did IKK α (Fig. 4). However, full-length I κ B α is phosphorylated by IKK α and IKK β with similar efficiencies. The Michaelis constants (K_m) of IKK α and IKK β toward full-length GST-I κ B α were 2.1 and 2.2 μ M, respectively (14).

In resting cells, cytoplasmic I κ B proteins are associated with NF- κ B dimers, and phosphorylation does not result in dissociation of this complex (2). Thus, the actual substrate for IKK is the I κ B-NF- κ B complex rather than free I κ B. Some stimuli—such as IL-1, which is one of the strongest NF- κ B activators—lead to prolonged activation of IKK that lasts 2 hours or more (4). Once I κ B α is phosphorylated and degraded (within 3 to 10 min), NF- κ B translocates to the nucleus and activates gene transcription (15), including that of the I κ B α gene (16). Newly synthesized free I κ B α accumulates within 60 min of the initial stimulus and translocates to the nucleus, where it binds DNA-bound NF- κ B to induce its shuttling to the cytoplasm (17). This process is important for termination of the NF- κ B response. But given the prolonged activation of IKK, it is puzzling how newly synthesized I κ B α escapes the IKK-induced degradation pathway. We therefore compared the efficiency with which IKK phosphorylated free and NF- κ B-bound I κ B α (18). At similar concentrations, I κ B α complexed with NF- κ B was a better substrate for IKK α or IKK β than was free I κ B α (Fig. 5). Kinetic analysis indicated that in the presence of NF- κ B, the K_m for I κ B α phosphorylation by IKK β decreased from 2.2 μ M to 1.4 μ M and the relative maximum initial velocity V_{max} was increased by a factor of 5 (14). Although I κ B β is not involved in rapid feedback inhibition of NF- κ B activity (19), its phosphorylation by IKK α or IKK β was also strongly enhanced by binding of NF- κ B (Fig. 5). These results, which show that IKK prefers NF- κ B-bound I κ B proteins, explain why newly synthesized free I κ B α is not phosphorylated and degraded in cells in which

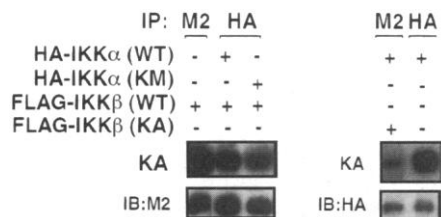


Fig. 3. Kinase activity of IKK heterodimers. Wild-type (FLAG)-IKK β was expressed together with either wild-type (HA)-IKK α or kinase-defective (HA)-IKK α (KM) in Sf9 cells. Wild-type IKK α was expressed by itself or together with kinase-defective FLAG-IKK β (KA). Extracts of cells infected with various combinations of the corresponding viruses, as indicated, were subjected to immunoprecipitation (IP) with either anti-HA or anti-FLAG (M2) as indicated. The kinase activity (KA) of each immune complex was examined with GST-I κ B α (1-54) as a substrate. Amounts of wild-type (FLAG)-IKK β or (HA)-IKK α proteins in the immune complexes were determined by immunoblotting (IB) with anti-FLAG (M2) or anti-HA, respectively. Exposure times for left and right panels were 1.5 and 5 hours, respectively.

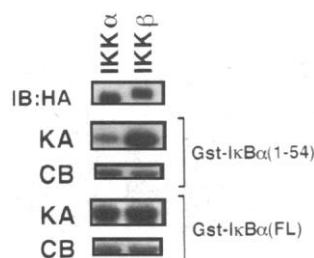


Fig. 4. Kinase activities of recombinant IKK α and IKK β . (HA)-IKK α and (HA)-IKK β were expressed separately in Sf9 cells (10) and immunoaffinity-purified on immobilized anti-HA. Immunoblot (IB) analysis shows that similar amounts of each protein were used to compare their kinase activities (KA) toward purified GST-I κ B α (1-54) and GST-I κ B α (FL) (full length). Coomassie blue (CB)-stained gels show the relative amounts of the two substrates.

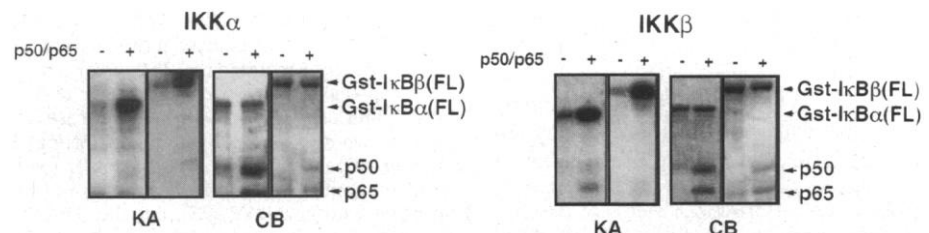


Fig. 5. Preferred phosphorylation by IKKs of I κ Bs bound to NF- κ B. The kinase activities (KA) of purified (HA)-IKK α (left panel) or (FLAG)-IKK β (right panel) toward free or NF- κ B-prebound GST-I κ B α (FL) and GST-I κ B β (FL) were measured. The NF- κ B heterodimer was composed of recombinant p50 and the Rel homology domain of p65 (18). Shown are the autoradiograms of the kinase assays (KA) and Coomassie blue (CB)-stained gels depicting the various substrates.

IKK remains active after the initial inductive stimulus.

The IKK α and IKK β protein kinases, and not another component of the IKK complex, appear to be directly responsible for I κ B phosphorylation. The minimal active IKK complex is apparently a dimer composed of IKK α or IKK β (or both). These experiments also provide an explanation for the mechanism underlying the termination of the NF- κ B activation response. Such inactivation is important because prolonged or chronic NF- κ B activation can result in inflammatory disorders (20).

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9. Transfer vectors were constructed using the baculovirus transfer vector pAcSGHis NT-B (Pharmingen). All inserts were prepared by polymerase chain reaction (PCR). HA-tagged IKK α (the HA epitope is fused in frame to the second amino acid of IKK α) was generated by PCR using the primers 5'-TACTAGCTC-GAGGATACCTTACGATGTTCTCTGATACGCTGAGCGGCCCGGGG-3' and 5'-AGCTATCGCGGCCGCTCATTTGTGTTAACCAAC-3'. This fragment was digested with Xho I and Not I and cloned into the corresponding sites of pAcSGHis NT-B. In the resulting vector, the (His)₆ tag is upstream of and in frame with the HA epitope. A similar strategy was used to generate the transfer vectors for (His)₆-HA-IKK β and (His)₆-FLAG-IKK β . Primers for FLAG-tagged and HA-tagged IKK β were 5'-AGCTATCGCGGCCGCGACTCAAGGACGACGATGACAAAGCTGGTCCACTTCC-3', 5'-AGCTAGCGCGGCCGCTACCTTACGATGTTCTGATACGCTAGCTGGTCACTTCC-3', and 5'-TGACTCCGGGTCTAGGCGCTGCTCC-3'. For the generation of recombinant viruses, we followed the manufacturer's (Pharmingen) recommendations for growth and maintenance of Sf9 cells, preparation of recombinant baculoviruses, and infection of the cells. For in vivo recombination, we transfected the transfer vectors and BaculoGold DNA into Sf9 cells using SuperFect (Qiagen). To prepare a control viral stock, we cotransfected Sf9 cells with unmodified pAcSGHis NT-B vector and BaculoGold DNA. After 5 days, culture supernatants were collected and amplified by two rounds of infection. For expression of both IKK α and IKK β , the corresponding recombinant viruses were mixed in various ratios before infection of Sf9 cells. The efficiency of infection was determined by indirect double immunofluorescence using antibodies to HA and FLAG.
10. Recombinant (His)₆-HA-IKK α and (His)₆-FLAG-IKK β proteins were produced in Sf9 cells after infection with high-titer recombinant viruses. The proteins were purified as described (7). Briefly, cell extracts were fractionated on Q-Sepharose and the fractions containing IKK were applied to an adenosine triphos-

phate (ATP) affinity column. Bound proteins were eluted with 20 mM ATP and applied to a Superose 6 gel filtration column. The fractions containing IKK (200 to 250 kD range) were then bound to Ni-Sepharose and washed extensively with AP buffer (500 mM NaCl, 1% Triton X-100, and 2 M urea) (7) and eluted with 200 mM imidazole. The eluted IKKs were either separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8% gel) and visualized by silver staining or further purified by immunoaffinity chromatography with anti-FLAG (M2)-coupled Sepharose for IKK β or anti-HA-coupled Sepharose for IKK α .

11. E. Zandi and Y. Chen, unpublished results. IKK α does not discriminate between full-length wild-type I κ B α and the I κ B α (A32/36) mutant as effectively as does IKK β . In addition to Ser³² and Ser³⁶, IKK α and to a lesser extent IKK β also phosphorylate sites close to the COOH-terminus of I κ B α (7).
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13. Purified IKK proteins were cross-linked in the presence of bovine serum albumin (BSA, 1 mg/ml) in AP buffer containing 300 mM NaCl, 1% Triton X-100, and 2 mM ethylene glycol-bis(succinimidylsuccinate) (EGS; Pierce) for 20 min at room temperature. The cross-linker was inactivated by addition of 500 mM tris-HCl (pH 8.0). Cross-linked proteins were either immunoprecipitated (as described below) before separation by SDS-PAGE (4.5% gel) or directly subjected to SDS-PAGE. Proteins were visualized by immunoblotting with specific antibodies. For immunoprecipitation, the cross-linked IKK preparations were divided in two; one portion was immunoprecipitated with anti-IKK α and the other with anti-IKK β . The immune complexes were separated by SDS-PAGE (4.5% gel) and proteins were transferred to a membrane and immunoblotted with an antibody to the other subunit than the one used for immunoprecipitation.
14. Various amounts of GST-I κ B α (full-length) or GST-

I κ B α (1-54) were incubated with purified (His)₆-HA-IKK α or (His)₆-FLAG-IKK β in kinase assay buffer (7) with 200 μ M [γ -³²P]ATP for 20 min at 30°C. The reactions were stopped by adding SDS loading buffer, and proteins were resolved by SDS-PAGE (10% gel). The phosphorylated I κ B bands were visualized and quantified by a phosphorimager (Bio-Rad). The amount of radioactivity in each band was plotted against the substrate concentration using a nonlinear regression program (SigmaPlot). The K_m values for each enzyme were determined from the corresponding plots.

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18. Full-length GST-I κ B α or GST-I κ B β (2 μ g) were incubated with an excess of purified NF- κ B dimers formed from recombinant p50(NF- κ B1) and the Rel homology domain of p65(RelA) [F. E. Chen, D. B. Huang, Y. Q. Chen, G. Ghosh, *Nature* **391**, 410 (1998)]. The GST-I κ B-p50-p65 complexes were separated from free NF- κ B by chromatography on glutathione-Sepharose 4B (Pharmacia). The ternary I κ B-NF- κ B complexes, whose formation was confirmed by SDS-PAGE and Coomassie blue staining, were washed with kinase buffer before kinase assay.
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Optimizing Gaze Control in Three Dimensions

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Horizontal and vertical movements of the human eye bring new objects to the center of the visual field, but torsional movements rotate the visual world about its center. Ocular torsion stays near zero during head-fixed gaze shifts, and eye movements to visual targets are thought to be driven by purely horizontal and vertical commands. Here, analysis of eye-head gaze shifts revealed that gaze commands were three-dimensional, with a separate neural control system for torsion. Active torsion optimized gaze control as no two-dimensional system could have, stabilizing the retinal image as quickly as possible when it would otherwise have spun around the fixation point.

The human eye rotates with three degrees of freedom: horizontally, vertically, and torsionally (1, 2). With few exceptions, theories of

vision and eye movement have ignored rotations about the line of sight. One reason is Listing's law (1, 3, 4), which states that ocular torsion stays near zero during head-fixed gaze shifts. This has been taken as confirmation that gaze is driven by purely horizontal and vertical commands. Here we show that Listing's law is just one mode of gaze control, valid only when the head is stationary.

When eye and head join forces to transport the gaze line, the logistics are complex. For one thing, the eye is quicker than the head—it reorients more swiftly when an interesting object

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