

MEKK1 activates both I κ B kinase α and I κ B kinase β

FRANK S. LEE*[†], ROBERT T. PETERS*[‡], LUAN C. DANG[‡], AND TOM MANIATIS*[§]

*Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138; and [‡]ProScript, Inc., 38 Sidney Street, Cambridge, MA 02139

Contributed by Tom Maniatis, May 21, 1998

ABSTRACT A critical step in the signal-induced activation of the transcription factor NF- κ B is the site-specific phosphorylation of its inhibitor, I κ B, that targets the latter for degradation by the ubiquitin–proteasome pathway. We have previously shown that mitogen-activated protein kinase/ERK kinase 1 (MEKK1) can induce both this site-specific phosphorylation of I κ B α at Ser-32 and Ser-36 *in vivo* and the activity of a high molecular weight I κ B kinase complex *in vitro*. Subsequently, others have identified two proteins, I κ B kinase α (IKK- α) and I κ B kinase β (IKK- β), that are present in a tumor necrosis factor α -inducible, high molecular weight I κ B kinase complex. These kinases are believed to directly phosphorylate I κ B based on the examination of the kinase activities of IKK immunoprecipitates, but more rigorous proof of this has yet to be demonstrated. We show herein that recombinant IKK- α and IKK- β can, in fact, directly phosphorylate I κ B α at Ser-32 and Ser-36, as well as homologous residues in I κ B β *in vitro*, and thus are bona fide I κ B kinases. We also show that MEKK1 can induce the activation of both IKK- α and IKK- β *in vivo*. Finally, we show that IKK- α is present in the MEKK1-inducible, high molecular weight I κ B kinase complex and treatment of this complex with MEKK1 induces phosphorylation of IKK- α *in vitro*. We conclude that IKK- α and IKK- β can mediate the NF- κ B-inducing activity of MEKK1.

The transcriptional activator protein NF- κ B plays a critical role in immune and inflammatory responses (for recent reviews, see refs. 1 and 2). NF- κ B is sequestered in the cytoplasm of most cell types by virtue of its association with the I κ B family of inhibitor proteins, which include I κ B α , I κ B β , and I κ B ϵ . Upon exposure to a wide variety of stimuli, including the proinflammatory cytokines tumor necrosis factor α (TNF- α) and interleukin 1, UV-irradiation, virus infection, bacterial lipopolysaccharide, and oxidative stress, the I κ B protein is phosphorylated at its N terminus. In the case of I κ B α , this occurs at Ser-32 and Ser-36; the corresponding residues in I κ B β are Ser-19 and Ser-23. This phosphorylation then targets I κ B for degradation by the ubiquitin–proteasome pathway, thereby liberating NF- κ B, which is then free to translocate to the nucleus and bind to DNA (3).

The mechanism by which the phosphorylation of the N terminus of I κ B occurs has recently been the subject of intense investigation (4, 5). A large, approximately 700-kDa multiprotein complex isolated from uninduced HeLa cells was originally demonstrated to phosphorylate I κ B α at Ser-32 and -36 (6). This complex could be activated *in vitro* by the mitogen-activated protein kinase kinase kinase (MAP3K) MEKK1, and the latter was shown to induce the site-specific phosphorylation of I κ B α *in vivo* (7). A model was proposed in which MEKK1 activates I κ B α kinase activity, which in turn leads to the phosphorylation of I κ B α . Inherent in this model was the

possibility that other MAP3Ks might also activate I κ B α kinase activity (7).

Subsequently, the MAP3K NF- κ B inducing kinase (NIK) was identified in a yeast two-hybrid screen based on its interaction with the TRAF2 component of the TNF- α receptor complex (8). The use of NIK as a bait in another yeast two-hybrid screen resulted in the cloning of the cDNA for I κ B kinase α (IKK- α) (9). IKK- β was then identified in a DNA database search by virtue of its homology to IKK- α (10). IKK- α and IKK- β cDNAs were independently cloned by using peptide sequences obtained from purified proteins (11–13). These proteins were isolated from high molecular weight complexes purified from TNF- α -treated HeLa cells. Immunoprecipitates of both IKK- α and IKK- β can phosphorylate I κ B α and I κ B β at the regulatory N-terminal Ser residues, and both IKKs can be activated by NIK (9–13). In addition, dominant negative versions of NIK, IKK- α , and IKK- β can inhibit TNF- α - and interleukin 1-induced NF- κ B activation (8–13).

These recent reports raise two important issues. (i) MEKK1 and NIK are MAP3Ks that share significant homology in their catalytic domains (8, 14). Thus, one issue is whether MEKK1, like NIK, can activate IKK- α and/or IKK- β . (ii) Previous reports did not demonstrate whether IKK- α and IKK- β can directly phosphorylate I κ B, because they used immunoprecipitates (as opposed to purified versions) of IKK- α and IKK- β to examine their kinase activity (9–13). Thus, IKK- α and IKK- β have not yet been shown to directly phosphorylate I κ B. Herein, we show that MEKK1 can activate both IKK- α and IKK- β *in vivo* and that recombinant versions of the latter two proteins can directly phosphorylate I κ B α and I κ B β *in vitro*. These findings lend support to a general model for NF- κ B activation that involves the sequential involvement of a MAP3K, an IKK, and an I κ B.

MATERIALS AND METHODS

Plasmids. pcDNA3-FlagJNK1, pcDNA3-NIK and pcDNA3-FlagNIK, and pRK-FlagIKK- α and - β and their dominant negative mutants, were gifts from Roger Davis, David Wallach, and David Goeddel, respectively, and have been described (8–10, 15). pCMV4-I κ B β and mutants were gifts from Dean Ballard (16). pBS-I κ B α (S32T/S36T) was constructed by overlapping PCR using pBS-I κ B α as a template (17). The sources of pCMV5-MEKK1 (which encodes the C-terminal 672 residues of MEKK1), pcDNA3-FlagMEKK1 Δ (which encodes the C-terminal 321 residues), (PRDII)₂CAT, (PRDIV)₆CAT, pCMV-lacZ, pBS-I κ B α , pBS-I κ B α (S32A),

Abbreviations: CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; IKK, I κ B kinase; JNK, c-Jun N-terminal kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MEKK1, mitogen-activated protein kinase/ERK kinase kinase 1; NIK, NF- κ B inducing kinase; TNF- α , tumor necrosis factor α .

A commentary on this article begins on page 9067.

[†]Present address: Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 218 John Morgan, Philadelphia, PA 19104.

[§]To whom reprint requests should be addressed. e-mail: maniatist@biohp.harvard.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/959319-6\$2.00/0 PNAS is available online at www.pnas.org.

pBS-I κ B α (S36A), pBS-I κ B α (S32A/S36A), and pRSET-I κ B α have been described (3, 7).

Tissue Culture and Transfection. HeLa cells were maintained as described (7). Transfections, performed in 3.5-cm-diameter wells, were conducted as described (18). Protein concentration, chloramphenicol acetyltransferase (CAT), and β -galactosidase measurements on cellular extracts were performed as described (7).

Western Blotting. Immunoprecipitates or aliquots of whole cell lysates were mixed with 2 \times SDS/PAGE loading buffer and then subjected to SDS/PAGE in 8% or 10% gels. Western blotting was then performed as described (7) by using anti-Flag, anti-JNK1 (C-17), or anti-IKK- α (H-744) polyclonal antibodies (Santa Cruz Biotechnology), anti-rabbit IgG-horseradish peroxidase conjugates (Santa Cruz Biotechnology), and Enhanced Chemiluminescence substrate (Amersham).

Preparation of Recombinant Proteins and Peptides. pFastBacHT-IKK- α and pFastBacHT-IKK- β were constructed by subcloning the coding sequence fragments of pRK-FlagIKK- α or pRK-FlagIKK- β , respectively, into pFastBacHTb (Life Technologies). Recombinant bacmids and baculovirus were prepared according to the manufacturer's instructions. (His)₆IKK- α and (His)₆IKK- β were isolated by using Ni-nitrilotriacetic acid agarose from baculovirus-infected Sf9 cells. (His)₆IKK- β was further purified by applying it to a Mono Q column equilibrated with 25 mM Bis-Tris, pH 6.3/10% glycerol and eluting it with a 0–1 M NaCl gradient in the same buffer. The concentrations of (His)₆IKK- α and (His)₆IKK- β were determined by SDS/PAGE followed by staining with Coomassie blue or silver and comparison with BSA standards. (His)₆MEKK1 Δ was prepared as described (7).

pGEX-I κ B α -(5–55), pGEX-I κ B α -(5–55) (S32A/S36A), and pRSET-I κ B α (S32A/S36A) were gifts from Jeremiah Hagler in the laboratory of T.M. These plasmids as well as pGEX-I κ B α -(5–55) (S32A), pGEX-I κ B α -(5–55) (S36A), and pGEX-I κ B α -(5–55) (S32T/S36T) were constructed by subcloning restriction enzyme fragments from appropriate pBS-I κ B α wild-type or mutant constructs into pGEX-5X-1 (Pharmacia) or pRSET (Invitrogen). pGEX-I κ B β -(1–46), pGEX-I κ B β -(1–46) (S19A), pGEX-I κ B β -(1–46) (S23A), pGEX-I κ B β -(1–46) (S19A/S23A), pGEX-I κ B β -(1–46) (S19T/S23T), and pRSET-I κ B β and pRSET-I κ B β (S19A/S23A) were constructed by subcloning restriction enzyme fragments from appropriate pCMV4-I κ B β wild-type or mutant constructs as above.

Glutathione S-transferase (GST) fusion proteins were purified from *Escherichia coli* HB101 transformed with the corresponding pGEX expression vectors, by using glutathione-agarose affinity chromatography (19). (His)₆-tagged proteins were purified by using Ni-NTA agarose from *E. coli* BL21(DE3)LysS transformed with the corresponding pRSET expression vectors. The concentrations of the various I κ B substrate proteins were determined by the Bradford method.

The following peptides were synthesized, isolated in greater than 95% purity (BioSynthesis), and dissolved in water: IKK- β -(171–187), KELDQGSLSFVGTQ; IKK- β -(171–187) (S177A/S181A), KELDQALCTAFVGTQ.

Protein Kinase Assays. When purified recombinant proteins were used as both enzyme and substrate, kinase assays were performed in 10 μ l of buffer A (20 mM Hepes, pH 7.6/20 mM β -glycerophosphate/0.1 mM sodium orthovanadate/10 mM MgCl₂/50 mM NaCl/1 mM DTT) containing 0.5 μ g of I κ B α or I κ B α fusion protein substrate, 50 μ M ATP, and 5 μ Ci of [γ -³²P]ATP (1 Ci = 37 GBq), and conducted at 30°C for 30 min. Reaction products were subjected to SDS/PAGE in 9 or 10% gels. When peptide substrates were used, the assay conditions were as above, except that 2 μ g of peptide was used, and reactions were performed for 60 min. Peptide reaction

products were subjected to Tris-Tricine 10–20% gradient SDS/PAGE (Bio-Rad).

Immunocomplex kinase assays for IKK and c-Jun N-terminal kinase (JNK) were performed by the methods of DiDonato *et al.* (11) and Derijard *et al.* (15), respectively, with minor modifications. Whole cell extracts were prepared by lysing cells in buffer B (20 mM Tris-HCl, pH 7.6/150 mM NaCl/25 mM β -glycerophosphate/2 mM EDTA/2 mM pyrophosphate/1 mM sodium orthovanadate/10% glycerol/1% Triton X-100/1 mM DTT) containing leupeptin (10 μ g/ml) and 1 mM phenylmethylsulfonyl fluoride. After centrifugation of the lysate at 16,000 \times *g* for 10 min at 4°C, the supernatant was incubated with 10 μ l of M2-agarose with end over end rotation for 1–2 hr at 4°C. For IKK assays, the resin was then washed once with buffer B, three times with buffer A containing 3 M urea, and then twice with buffer A. IKK reactions were initiated by the addition of 10 μ l of buffer A containing 0.5 μ g of GST-I κ B α -(5–55), 200 μ M ATP, and 5 μ Ci of [γ -³²P]ATP and performed at 30°C for 15–45 min. For JNK assays, the resin was washed three times with buffer B then once with buffer A. JNK reactions were initiated by the addition of 10 μ l of buffer A containing 0.36 μ g of GST-c-Jun-(1–79) (GST fused to residues 1–79 of c-Jun; Santa Cruz Biotechnology), 50 μ M ATP, and 5 μ Ci of [γ -³²P]ATP, and performed at 30°C for 20 min. Kinase reaction products were subjected to SDS/PAGE in 8 or 10% gels. Kinase activities were quantified by using a phosphorimager.

I κ B α mobility shift assays were performed as described (7), except that creatine phosphokinase and phosphocreatine were omitted.

Purification of I κ B α Kinase Complex. I κ B α kinase complex was purified as described (6) with minor modifications. Cytoplasmic extracts (S100) from uninduced HeLa cells were subjected to precipitation with 30% ammonium sulfate, resuspended, dialyzed against buffer C (50 mM Tris-HCl, pH 7.5/5% glycerol/0.5 mM DTT/0.1 mM phenylmethylsulfonyl fluoride), and applied to a Mono Q HiTrap column (Pharmacia). The column was washed with buffer C containing 0.2 M KCl and material was eluted with buffer C containing 0.3 M KCl. Fractions containing I κ B α kinase activity were then pooled, concentrated, and applied first to a Superdex-200 column (Pharmacia) equilibrated in buffer C containing 150 mM NaCl and then to a BioQ2 column (Bio-Rad), and material was eluted with a linear gradient of 150–405 mM NaCl in buffer C. Fractions containing activity were dialyzed against buffer D (20 mM Tris-HCl, pH 7.5/20 mM NaCl/50% glycerol/0.5 mM DTT/0.1 mM phenylmethylsulfonyl fluoride/leupeptin at 10 μ g/ml).

IKK- α Immunoprecipitations. I κ B α kinase complex was incubated in 110 μ l of buffer D containing 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 2.5 μ M okadaic acid, 10 mM MgCl₂, 10 μ M ATP, and 25 μ Ci of [γ -³²P]ATP at 30°C for 30 min in the absence or presence of 40 ng of MEKK1 Δ . Reactions were then incubated with either anti-Stat1 (E-23) or anti-IKK- α (H-744) polyclonal antibodies (Santa Cruz Biotechnology) prebound to protein G-agarose in 600 μ l of buffer E (50 mM Tris-HCl, pH 8.0/0.5% sodium deoxycholate/1% Nonidet P-40/0.1% SDS) with rocking for 1 hr at 4°C. Resins were washed twice with buffer E, twice with buffer A containing 3 M urea, and then twice with buffer E. Immunoprecipitated material was eluted with 2 \times SDS loading buffer and subjected to SDS/PAGE in 8% gels. Reactions performed in the presence of [γ -³²P]ATP were analyzed by autoradiography, and parallel reactions performed in its absence were analyzed by Western blotting.

RESULTS

Phosphorylation of I κ B α and I κ B β by IKK- α and IKK- β *In Vitro*. To examine whether IKK- α and IKK- β can directly

phosphorylate the critical Ser residues at the N terminus of I κ B, polyhistidine-tagged IKK- α and IKK- β were purified as recombinant proteins from baculovirus-infected insect cells by using Ni-agarose chromatography (Fig. 1A). When IKK- α (Fig. 1C) or IKK- β (Fig. 1D) were incubated with GST fused to residues 5–55 of I κ B α [GST-I κ B α (5–55)] or residues 1–46 of I κ B β [GST-I κ B β (1–46)] in the presence of [γ - 32 P]ATP, phosphorylation of the I κ B fusion proteins was readily detected (Fig. 1C and D, lanes 1 and 6). A double mutation of either the I κ B α Ser-32 and -36 to Ala (lane 4) or the I κ B β Ser-19 and -23 to Ala (lane 9) virtually abolished phosphorylation, indicating that phosphorylation of the wild-type proteins occurred at these residues.

To further define the targets of phosphorylation, I κ B α and I κ B β fusion proteins bearing single point mutations at these residues were examined as substrates. Both IKK- α and IKK- β efficiently phosphorylated all of these substrates (Fig. 1C and D, lanes 2, 3, 7, and 8), implying that both IKKs phosphorylate both Ser-32 and -36 of I κ B α and Ser-19 and -23 of I κ B β . Mutation of these residues to Thr resulted in substrates that are poorer than the wild-type version (Fig. 1C and D, lanes 5 and 10); overexpressed I κ B α s bearing the same mutations at either site displayed impaired TNF- α -induced degradation *in vivo* (20). The marked preference reported for IKK- α for Ser-23 over Ser-19 of I κ B β (9) was not observed here (Fig. 1C, compare lanes 7 and 8). Whether the discrepancy in results is due to differences in the source of IKK- α [transfected mammalian 293 cells (9) vs. baculovirus-infected Sf9 cells (present report)] or differences in the substrates used [I κ B β (1–311) vs. GST-I κ B β (1–46)] is not clear.

In addition to the GST fusion proteins containing the N terminus of I κ B, we also examined full-length I κ B α and I κ B β as substrates. As with the GST fusion proteins, the full-length proteins were both substrates for both IKK- α and IKK- β (Fig. 1F, lower bands of odd numbered lanes). In contrast to the GST fusion proteins, however, double mutations of I κ B α Ser-32 and -36 to Ala or I κ B β Ser-19 and -23 to Ala only partially diminished 32 P incorporation into the substrates (lower bands of even numbered lanes). This result indicates that at least *in vitro*, IKK- α and IKK- β can phosphorylate I κ B residues distinct from these N-terminal residues. Studies conducted with IKK- α immunoprecipitates suggest that additional I κ B α phosphoacceptor sites reside between residues 263 and

315 (11). Both IKK- α and IKK- β displayed autophosphorylating activity (Fig. 1F, upper bands).

To address the possibility that the purified IKK- β is contaminated with an insect cell kinase activity, the recombinant IKK- β was further purified by Mono Q chromatography (Fig. 1B) and assayed for kinase activity (Fig. 1E). This IKK- β phosphorylated the wild-type and mutant I κ B α and I κ B β fusion proteins with a substrate specificity essentially the same as that of the less-purified IKK- β (Fig. 1, compare D and E). A 30-kDa polypeptide still copurified with IKK- β (Fig. 1B, asterisk). However, assay of other fractions containing this polypeptide but not IKK- β revealed no detectable I κ B kinase activity (data not shown). We conclude that the observed activity derives from the purified recombinant IKK- β .

Activation of IKK- α and IKK- β by MEKK1 *in Vivo*. To address whether MEKK1 can activate either IKK- α or IKK- β activity, HeLa cells were cotransfected with expression vectors for either IKK- α or IKK- β and expression vectors for MEKK1, NIK, or expression vector alone. The IKK was then immunoprecipitated and assayed for kinase activity against GST-I κ B α (5–55). As shown in Fig. 2A, overexpressed and immunoprecipitated IKK- α displays barely detectable I κ B α kinase activity under the conditions used (Fig. 2A, lane 1, lower band). This activity can be augmented by coexpression with NIK (Fig. 2A, lane 3) (9) and, importantly, also by coexpression with MEKK1 (Fig. 2A, lane 2). Overexpressed and immunoprecipitated IKK- β displays a substantially higher basal I κ B α kinase activity (Fig. 2A, lane 4, lower band) relative to IKK- α , as noted by others (10, 12, 13). Significantly, this activity can also be augmented by coexpression with either MEKK1 or NIK (Fig. 2A, lanes 5 and 6). In all cases, IKK expression levels were not significantly affected by this coexpression (Fig. 2B). Immunoprecipitated IKK- α has relatively weak I κ B α kinase activity with the GST-I κ B α (5–55) substrate, whereas immunoprecipitated IKK- β has relatively strong activity (Fig. 2A, compare lower bands of lanes 1 and 4). In addition, both immunoprecipitated proteins display autophosphorylating activity (Fig. 2A, upper bands).

Stimuli such as proinflammatory cytokines and UV-irradiation not only activate the NF- κ B pathway but also the JNK (also known as the stress-activated protein kinase) pathway (15, 21). Therefore, MEKK1 and NIK were further compared with respect to their activation of these pathways in

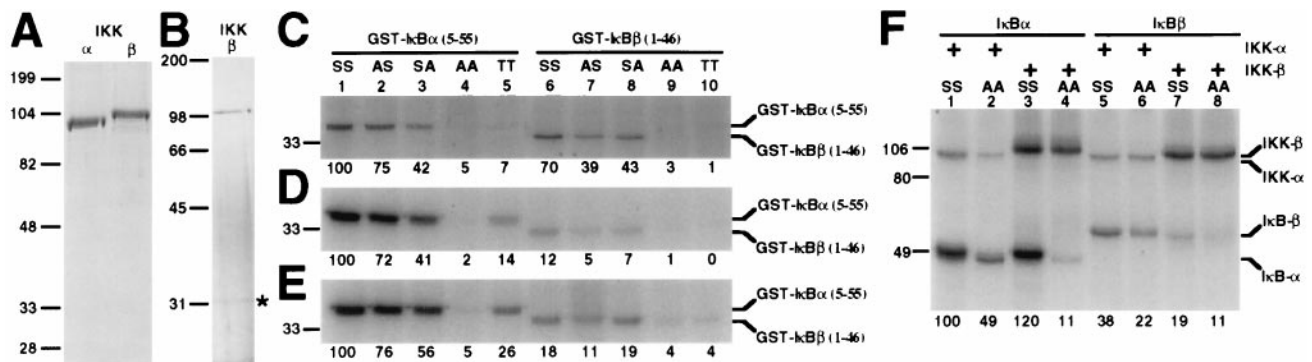


FIG. 1. Phosphorylation of I κ B α and I κ B β by recombinant IKK- α and IKK- β . (His) $_6$ -tagged IKK- α (A) and IKK- β (A and B) were purified from baculovirus-infected Sf9 cells with Ni-NTA agarose chromatography (A) and further by Mono Q chromatography (B), and 500 (A) or 20 ng (B) was subjected to SDS/PAGE in 8 or 10% gels and stained with Coomassie blue (A) or silver (B). An asterisk in B denotes a polypeptide copurifying with IKK- β . (C–E) GST-I κ B α (5–55) (0.5 μ g) or GST-I κ B β (1–46) (0.5 μ g) bearing the indicated residues was incubated in the presence of [γ - 32 P]ATP with recombinant IKK- α (10 ng) (C) or IKK- β (1 ng) (D and E) purified by Ni-agarose chromatography (C and D) or further by Mono Q chromatography (E). The first and second residues correspond to positions 32 or 36 of I κ B α , respectively, or to positions 19 and 23 of I κ B β , respectively. Reaction products were subjected to SDS/PAGE in 10% gels and analyzed by autoradiography. (F) (His) $_6$ I κ B α (0.5 μ g), (His) $_6$ I κ B α (S32A/S36A) (0.5 μ g), (His) $_6$ I κ B β (0.5 μ g), or (His) $_6$ I κ B β (S19A/S23A) (0.5 μ g) was incubated in the presence of [γ - 32 P]ATP with 10 ng of recombinant IKK- α or IKK- β purified by Ni-agarose chromatography. Reaction products were subjected to SDS/PAGE in 9% gels and analyzed by autoradiography. The slower migration of wild-type as compared with S32A/S36A (His) $_6$ I κ B α reflects phosphorylation at Ser-32 and -36 (6). (A–F) Molecular mass markers (in kDa) are indicated to the left, and the positions of IKK- α , IKK- β , and the I κ B α and I κ B β substrates are indicated to the right. The relative levels of 32 P incorporation into the I κ B substrates are indicated below the gels.

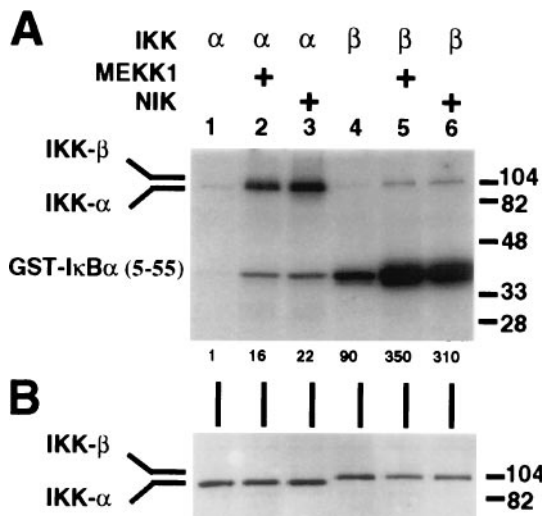


FIG. 2. Activation of IKK- α and IKK- β by MEKK1 and NIK. HeLa cells were transfected with 1 μ g of pRK-FlagIKK- α or 1 μ g of pRK-FlagIKK- β and 6 μ g of pCMV5-MEKK1, 6 μ g of pcDNA3-NIK, or 6 μ g of pcDNA3. Cells were harvested 42 hr after transfection, and IKK- α or IKK- β was immunoprecipitated with anti-Flag M2-agarose. (A) IKK immunocomplexes were assayed for kinase activity by incubation with 0.5 μ g of GST-I κ B α (5-55) in the presence of [γ - 32 P]ATP. Reaction products were subjected to SDS/PAGE in 10% gels and analyzed by autoradiography. The relative level of 32 P incorporation into the GST-I κ B α (5-55) substrate is indicated below the gel. (B) IKK immunocomplexes were subjected to SDS/PAGE in 8% gels, transferred to nitrocellulose membrane, and probed with anti-Flag polyclonal antibodies. (A and B) The positions of IKK- α , IKK- β , and GST-I κ B α (5-55) are indicated to the left, and molecular mass markers (in kDa) are indicated to the right.

HeLa cells. Two different assays were used. The first measured the kinase activity of coexpressed and immunoprecipitated IKK (as above) or JNK. The second measured the activity of a reporter gene for either the NF- κ B or JNK pathways. The reporter gene for the NF- κ B pathway is driven by two copies of the interferon β (IFN- β) enhancer PRDII element, which binds to NF- κ B (22, 23). That for the JNK pathway is driven by six copies of the IFN- β enhancer PRDIV element, which binds to an ATF-2/c-Jun heterodimer (24); both ATF-2 and c-Jun contain transcriptional activation domains that can be phosphorylated and activated by the JNK pathway (25). As shown in Fig. 3A, MEKK1 and NIK display similar dose-response curves with respect to their activation of coexpressed IKK- α or IKK- β . However, NIK is a substantially more potent activator of the NF- κ B reporter gene than MEKK1, with substantial activation seen with a NIK dose (50 ng) two orders of magnitude lower than that required to detect MEKK1-induced activation (5 μ g) (Fig. 3C). We note, however, that increasing levels of reporter gene activity could be detected with intermediate levels of transfected MEKK1 DNA (between 0.5 and 5 μ g; data not shown). NIK does not activate either coexpressed JNK or the JNK reporter gene, in contrast to MEKK1, which activates both (Fig. 3B and D), consistent with previous reports (7, 26–28).

Inhibition of MEKK1-Induced NF- κ B Activation *in Vivo* by Dominant Negative IKK- α and Dominant Negative IKK- β . To examine whether IKK- α or IKK- β mediate MEKK1-induced activation of NF- κ B, HeLa cells were cotransfected with the NF- κ B reporter gene, expression vectors for MEKK1 Δ (which contains the kinase domain of MEKK1), NIK, or the expression vector alone, and expression vectors for catalytically inactive IKK- α , IKK- β , or the expression vector alone. As shown in Fig. 4, both MEKK1 Δ and NIK activate the NF- κ B reporter gene, as expected. Under conditions where both dominant negative IKK- α and IKK- β inhibit NIK-induced

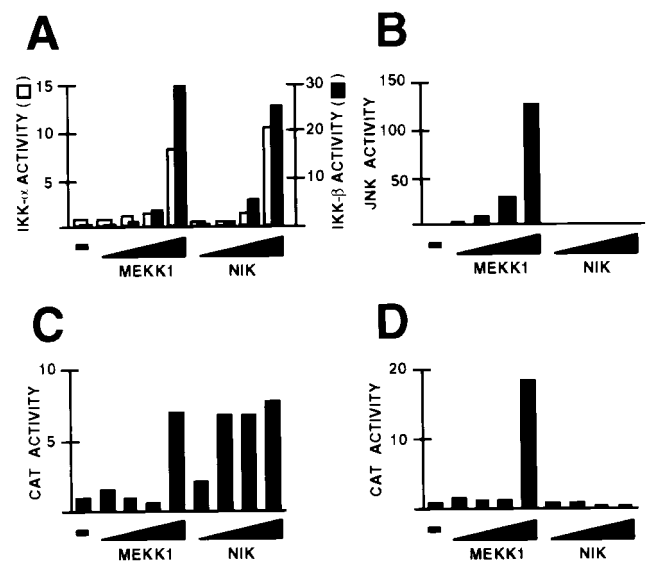


FIG. 3. Effects of MEKK1 and NIK on the NF- κ B and JNK pathways. HeLa cells were transfected with 2 μ g of pCMV-lacZ; 1 μ g of pRK-FlagIKK- α (A), 1 μ g of pRK-FlagIKK- β (A), 1 μ g of pcDNA3-FlagJNK1 (B), 3 μ g of (PRDII) $_2$ CAT (C), or 3 μ g of (PRDIV) $_6$ CAT (D); and 5, 50, 500, or 5000 ng of either pCMV5-MEKK1 or pcDNA3-NIK. The total DNA dose was brought up to 8 (A and B) or 10 (C and D) μ g with pcDNA3. Cells were harvested 40–42 hr after transfection. (A and B) Immunocomplex kinase assays. Whole cell extracts, the volumes of which were normalized for β -galactosidase activity, were subjected to immunoprecipitation with anti-Flag M2-agarose. Immunocomplexes were assayed for IKK (A) or JNK (B) activities by incubation with GST-I κ B α (5-55) or GST-cJun(1-79), respectively, in the presence of [γ - 32 P]ATP. Western blots performed on aliquots of whole cell extracts revealed that comparable amounts of protein kinase were subjected to immunoprecipitation in each series of experiments (data not shown). Activities were normalized to that of IKK- α , IKK- β , or JNK alone, each of which was assigned a value of 1. (C and D) CAT assays. Activities were normalized to protein concentrations of cell extracts. Shown is a representative result from three experiments.

activation of the NF- κ B reporter gene, both mutants also potentially inhibit MEKK1 Δ -induced activation of this same reporter gene, providing further evidence that IKK- α and IKK- β can mediate MEKK1-induced NF- κ B activation *in vivo*. Similar results were reported with 293 cells (29).

Induced Phosphorylation of IKK by MEKK1. In previous studies, MEKK1 was shown to activate a 700-kDa I κ B kinase

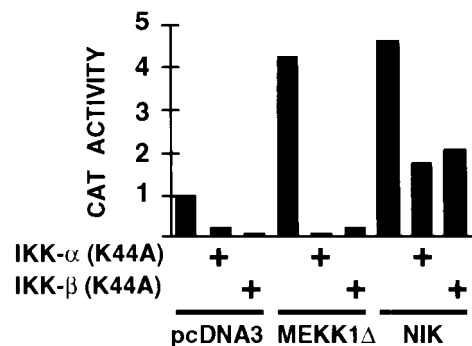


FIG. 4. Inhibition of MEKK1- and NIK-induced NF- κ B activation by dominant negative IKK- α or dominant negative IKK- β . HeLa cells were transfected with 3 μ g of (PRDII) $_2$ CAT; 4 μ g of pcDNA3-FlagMEKK1 Δ , 0.1 μ g of pcDNA3-FlagNIK, or 4 μ g of pcDNA3; and 4 μ g of pRK-FlagIKK- α (K44A), pRK-FlagIKK- β (K44A), or pcDNA3. The total DNA dose was brought up to 11 μ g with pcDNA3. Cells were harvested 40 hr after transfection. CAT activities were normalized to protein concentrations of cell extracts. Shown is a representative result from three experiments.

complex isolated from uninduced cells (7), but these studies were carried out prior to the discovery of IKK- α and IKK- β . We therefore carried out Western blotting experiments with anti-IKK- α antibodies and found that IKK- α is indeed present in this complex (data not shown). In addition, the MEKK1-induced I κ B α kinase activity could be immunoprecipitated with the anti-IKK- α antibodies (data not shown). Finally, a Western blot of the complex immunoprecipitated with anti-IKK- α antibodies revealed the presence of IKK- α (Fig. 5A, lane 3). To determine whether IKK- α in this complex is phosphorylated in response to MEKK1, the complex was incubated in the presence of [γ - 32 P]ATP with or without MEKK1 Δ , subjected to immunoprecipitation with anti-IKK- α antibodies, and examined by autoradiography. In the absence of MEKK1 Δ , there was marginal incorporation of 32 P into IKK- α (Fig. 5B, lane 3). By contrast, in the presence of MEKK1 Δ , this incorporation was markedly enhanced (lane 4). Importantly, the activity of the purified I κ B kinase complex was dependent on MEKK1, as demonstrated by incubating the kinase complex with 35 S-labeled Flag-I κ B α in the absence or presence of MEKK1 Δ and detecting the phosphorylation-dependent mobility shift of I κ B α (Fig. 5C, compare lanes 3 and 4). This mobility shift was abolished by a S32A/S36A double mutation of I κ B α (data not shown).

An additional slower-migrating species that coimmunoprecipitates with IKK- α is detectable upon MEKK1 Δ treatment (Fig. 5B, lane 4, asterisk). This species is most likely IKK- β because it has been previously shown to (i) display a slightly slower mobility than IKK- α by SDS/PAGE (10, 12, 13), (ii) copurify with IKK- α as a high molecular weight complex from TNF- α -treated HeLa cells (11–13), and (iii) interact with IKK- α *in vitro* and *in vivo* (10, 12, 13). Further studies are necessary to substantiate this proposal.

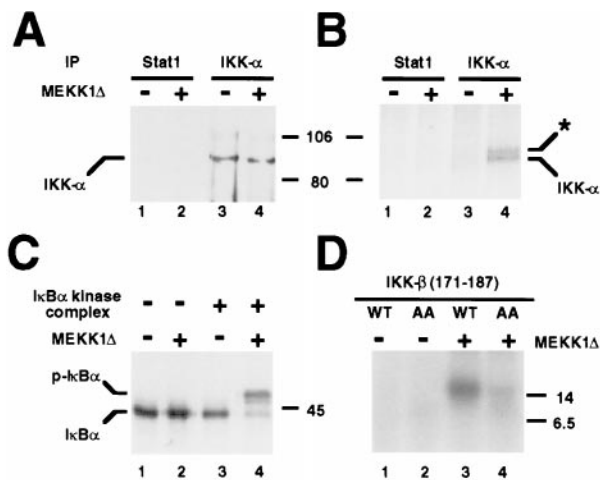


Fig. 5. MEKK1-induced phosphorylation of IKK. (A and B) I κ B α kinase complex was incubated with or without 40 ng MEKK1 Δ , in the absence (A) or presence (B) of [γ - 32 P]ATP. Reactions were then immunoprecipitated (IP) with either anti-Stat1 or anti-IKK- α antibodies and analyzed by Western blotting (A) using anti-IKK- α antibodies or by autoradiography (B). The position of IKK- α is as indicated and the position of a slower migrating species is denoted by an asterisk (see Results). (C) I κ B α kinase complex was incubated with 35 S-labeled *in vitro*-translated Flag-I κ B α in the absence or presence of 40 ng MEKK1 Δ . Reaction products were subjected to SDS/PAGE in 9% gels and detected by autoradiography. The positions of Flag-I κ B α (I κ B α) and phospho-Flag-I κ B α (P-I κ B α) are indicated to the left. (D) IKK- β (171–187) (2 μ g) or IKK- β (171–187) (S177A/S181A) (2 μ g) peptide was incubated with or without 100 ng of recombinant MEKK1 Δ in the presence of [γ - 32 P]ATP. Reaction products were subjected to gradient SDS/PAGE in 10–20% gels and analyzed by autoradiography. The positions of molecular mass markers (in kDa) are indicated to the left (B) or right (A, C, and D).

The experiment shown in Fig. 5B reveals MEKK1 Δ -dependent 32 P incorporation into IKK- α but does not assess the relative contributions to this by IKK- α autophosphorylation vs. phosphorylation by another kinase, such as MEKK1 Δ . The latter is a distinct possibility, particularly because both IKK- α and IKK- β possess canonical MAP2K activation loop motifs (SXXXS) (13). Indeed, mutation of two IKK- β Ser residues (Ser-177 and Ser-181) in this motif to Glu results in a constitutively active kinase; conversely, their mutation to Ala results in a dominant negative inhibitor of NF- κ B activation (13). To examine whether this activation loop might be directly phosphorylated by MEKK1, we synthesized a peptide that contains the activation loop of IKK- β , residues 171–187. As shown in Fig. 5D, when recombinant MEKK1 Δ is incubated with this peptide in the presence of [γ - 32 P]ATP, phosphorylation of the peptide is observed (Fig. 5D, lane 3). Replacement of Ser-177 and Ser-181 with Ala substantially reduces phosphorylation (Fig. 5D, lane 4), indicating that phosphorylation of the wild-type peptide has occurred at either or both of these residues. Ser-176, the homologue of IKK- β Ser-177 in IKK- α , has been proposed to be the major site of IKK- α phosphorylation by NIK (30).

DISCUSSION

Recombinant IKK- α and IKK- β were purified from baculovirus-infected insect cells (Fig. 1 A and B). Both proteins directly phosphorylate Ser-32 and -36 of I κ B α and Ser-19 and -23 of I κ B β *in vitro* and are, therefore, *bona fide* I κ B kinases. That being said, given the homology between the NF- κ B pathway in mammals and the Dif immune response pathway in *Drosophila* (31), it is conceivable that *in vivo* these overexpressed IKKs (and by analogy, the insect cell IKK homologues) might exist as high molecular weight complexes in insect cells, just as they do in mammalian cells (11–13). Furthermore, association with other proteins might be necessary to achieve activation. Although the data imply that IKK- α /IKK- β heterodimerization is not obligatory for activity, it is conceivable that heterodimerization might modulate IKK- α or IKK- β activity and/or substrate specificity.

Recombinant IKK- α and IKK- β purified from baculovirus-infected Sf9 cells are active enzymes (Fig. 1). One possible reason for this is that overexpression of these kinases in insect cells, just as with the overexpression of certain kinases in mammalian cells, results in their constitutive activation. This, in turn, could be due to trans- or autophosphorylation or, perhaps, protein expression levels that exceed the capacity of endogenous negative regulatory factors. Another possibility is that the baculovirus infection itself might result in IKK activation, because virus infection is a well known activator of NF- κ B in mammalian cells (22, 23, 32).

NIK is substantially more potent than MEKK1 in activating an NF- κ B reporter gene (Fig. 3C), as observed (28). However, NIK and MEKK1 are equally effective in activating coexpressed IKK- α or IKK- β (Fig. 3A). The reason for this discrepancy is presently not clear. One possibility is that NIK might possess NF- κ B inducing activities distinct from its IKK-inducing activity. However, it should be noted that catalytically inactive NIK does not activate NF- κ B (8), implying that if this is indeed the case, then NIK phosphorylates a substrate in addition to IKK in the NF- κ B pathway. Another possibility is that overexpressed IKK might respond less sensitively to coexpressed NIK (or MEKK1) than endogenous IKK. In support of the latter possibility, we find that overexpressed IKK- α or IKK- β can inhibit NIK- or MEKK1-induced activation of an NF- κ B reporter gene (data not shown). Hence, overexpression of IKK itself might perturb the NF- κ B signaling pathway.

These findings suggest limitations to experiments that examine the relative potency of overexpressed kinases such as

NIK and MEKK1 in activating particular pathways. Just to highlight this general point, overexpressed IKK- α has substantially lower I κ B α kinase activity than IKK- β (Fig. 2A) (10, 12, 13), yet antisense expression vectors and/or dominant negative versions of IKK- α and IKK- β indicate that both are essential for cytokine-induced activation of NF- κ B (9–13). Thus, the potency of kinases observed in transfection experiments may not necessarily provide an accurate indication of their relative roles under physiological conditions.

We have previously proposed that MEKK1 is a coordinate activator of the NF- κ B and JNK pathways (7). The fact that MEKK1 displays similar titration curves with the NF- κ B and JNK reporter genes (Fig. 3 C and D) is consistent with this proposal; in both cases, significant activation of the reporter gene is observed at only the highest MEKK1 dose used (5 μ g). We note that detectable JNK activation was observed at the lowest MEKK1 dose used (5 ng; Fig. 3B), raising the possibility that MEKK1 preferentially activates the JNK pathway (28). However, the activation observed at this dose is modest with respect to that seen at the highest dose (5 μ g), and furthermore, no saturation of either IKK (Fig. 3A) or JNK (Fig. 3B) activities is seen with the MEKK1 dose range used. Thus, this provides further evidence that the two pathways can be coordinately activated by MEKK1.

A model for NF- κ B activation (7) can now be extended to include the recently cloned NIK, IKK- α , and IKK- β . The essence of this model is a sequential involvement of a MAP3K (such as MEKK1 or NIK), an IKK (such as IKK- α or IKK- β), and an I κ B. Although MEKK1 can directly phosphorylate a peptide containing the activation loop motif of IKK- β (Fig. 5D), it still remains to be rigorously determined whether MEKK1 and NIK directly activate IKK- α and IKK- β , and in fact, whether MEKK1 and NIK act in a functionally parallel manner, as would be predicted from the homology in their catalytic domains. Nonetheless, one implication of this model is that there might be additional MAP3Ks that activate IKK activity. These considerations suggest the possibility that at least some of the extremely large number of stimuli that activate NF- κ B signal through distinct MAP3Ks.

This model, however, does not rule out other modes of regulation of IKK activity. Indeed, the 700-kDa I κ B α kinase complex originally purified from uninduced HeLa cells can be activated *in vitro* by ubiquitination (6), suggesting that this particular I κ B α kinase complex might serve to integrate phosphorylation- and ubiquitination-dependent signals leading to the activation of NF- κ B. In addition, mutations in a helix-loop-helix domain of IKK- α severely impair its catalytic activity, suggesting that as yet to be identified protein-protein interactions might also regulate activity (12).

Note Added in Proof. Yin *et al.* (33) have recently shown that the human T cell leukemia virus type 1 (HTLV-1) transactivator protein Tax activates NF- κ B through MEKK1.

We are grateful to Drs. Roger Davis, David Wallach, David Goeddel, Dean Ballard, and Jeremiah Hagler for gifts of plasmids. We also thank Dr. Vito Palombella and members of the Maniatis laboratory for helpful discussions and critical readings of the manuscript. F.S.L. was supported by a postdoctoral research fellowship for physicians from the Howard Hughes Medical Institute. R.T.P. was supported by National Institutes of Health Biology Predoctoral Training Grant 5T32 GM07598. This work was supported by National Institutes of Health Grant AI20642 to T.M. and by ProScript, Inc.

- Lee, F. S., Chen, Z. J. & Maniatis, T. (1998) in *Transcriptional Regulation of Endothelial Cell Adhesion Molecules*, ed. Collins, T. (Landes, Austin, TX), in press.
- May, M. J. & Ghosh, S. (1998) *Immunol. Today* **19**, 80–88.
- Chen, Z. J., Hagler, J., Palombella, V., Melandri, F., Scherer, D., Ballard, D. & Maniatis, T. (1995) *Genes Dev.* **9**, 1586–1597.
- Maniatis, T. (1997) *Science* **278**, 818–819.
- Stankovski, I. & Baltimore, D. (1997) *Cell* **91**, 299–302.
- Chen, Z. J., Parent, L. & Maniatis, T. (1996) *Cell* **84**, 853–862.
- Lee, F. S., Hagler, J., Chen, Z. J. & Maniatis, T. (1997) *Cell* **88**, 213–222.
- Malinin, N. L., Boldin, M. P., Kovalenko, A. V. & Wallach, D. (1997) *Nature (London)* **385**, 540–544.
- Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. & Rothe, M. (1997) *Cell* **90**, 373–383.
- Woronicz, J. D., Gao, X., Cao, Z., Rothe, M. & Goeddel, D. V. (1997) *Science* **278**, 866–869.
- DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. & Karin, M. (1997) *Nature (London)* **388**, 548–554.
- Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M. & Karin, M. (1997) *Cell* **91**, 243–252.
- Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A. & Rao, A. (1997) *Science* **278**, 860–866.
- Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. & Johnson, G. L. (1993) *Science* **260**, 315–319.
- Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. (1994) *Cell* **76**, 1025–1037.
- McKinsey, T. A., Brockman, J. A., Scherer, D. C., Al-Murrani, S. W., Green, P. L. & Ballard, D. W. (1996) *Mol. Cell. Biol.* **16**, 2083–2090.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989) *Current Protocols in Molecular Biology* (Wiley, New York).
- Thanos, D. & Maniatis, T. (1992) *Cell* **71**, 777–789.
- Smith, D. B. & Johnson, K. S. (1988) *Gene* **67**, 31–40.
- DiDonato, J. A., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. & Karin, M. (1996) *Mol. Cell. Biol.* **16**, 1295–1304.
- Kyriakis, J. M., Banerjee, P., Nikolaki, E., Dai, T., Ruble, E. A., Ahmad, M. F., Avruch, J. & Woodgett, J. R. (1994) *Nature (London)* **369**, 156–160.
- Visvanathan, K. V. & Goodbourn, S. (1989) *EMBO J.* **8**, 1129–1138.
- Lenardo, M. J., Fan, C.-M., Maniatis, T. & Baltimore, D. (1989) *Cell* **57**, 287–294.
- Du, W., Thanos, D. & Maniatis, T. (1993) *Cell* **74**, 887–898.
- Gupta, S., Campbell, D., Derijard, B. & Davis, R. J. (1995) *Science* **267**, 389–393.
- Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L. & Karin, M. (1994) *Science* **266**, 1719–1723.
- Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R. & Templeton, D. J. (1994) *Nature (London)* **372**, 798–800.
- Song, H. Y., Regnier, C. H., Kirschning, C. J., Ayres, T. M., Goeddel, D. V. & Rothe, M. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 9792–9796.
- Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H. & Okumura, K. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3537–3542.
- Ling, L., Cao, Z. & Goeddel, D. V. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3792–3797.
- Wu, L. P. & Anderson, K. V. (1998) *Nature (London)* **392**, 93–97.
- Fujita, T., Miyamoto, M., Kimura, Y., Hammer, J. & Taniguchi, T. (1989) *Nucleic Acids Res.* **17**, 3335–3346.
- Yin, M.-J., Christerson, L. B., Yamamoto, Y., Kwak, Y.-T., Xu, S., Mercurio, F., Barbosa, M., Cobb, M. & Gaynor, R. (1998) *Cell* **93**, 875–884.