MOLECULAR AND CELLULAR BIOLOGY, Dec. 1998, p. 7336–7343 Vol. 18, No. 12
0270-7306/98/$04.00+0

Coordinate Regulation of IκB Kinases by Mitogen-Activated Protein Kinase Kinase Kinase 1 and NF-κB-Inducing Kinase

SHINO NEMOTO,[1] JOSEPH A. DIDONATO,[2] AND ANNING LIN[1]*

Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama 35294,[1] and Department of Cancer Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195[2]

Received 1 May 1998/Returned for modification 5 June 1998/Accepted 16 September 1998

IκB kinases (IKKα and IKKβ) are key components of the IKK complex that mediates activation of the transcription factor NF-κB in response to extracellular stimuli such as inflammatory cytokines, viral and bacterial infection, and UV irradiation. Although NF-κB-inducing kinase (NIK) interacts with and activates the IKKs, the upstream kinases for the IKKs still remain obscure. We identified mitogen-activated protein kinase kinase kinase 1 (MEKK1) as an immediate upstream kinase of the IKK complex. MEKK1 is activated by tumor necrosis factor alpha (TNF-α) and interleukin-1 and can potentiate the stimulatory effect of TNF-α on IKK and NF-κB activation. The dominant negative mutant of MEKK1, on the other hand, partially blocks activation of IKK by TNF-α. MEKK1 interacts with and stimulates the activities of both IKKα and IKKβ in transfected HeLa and COS-1 cells and directly phosphorylates the IKKs in vitro. Furthermore, MEKK1 appears to act in parallel to NIK, leading to synergistic activation of the IKK complex. The formation of the MEKK1-IKK complex versus the NIK-IKK complex may provide a molecular basis for regulation of the IKK complex by various extracellular signals.

The transcription factor NF-κB regulates gene expression in response to various extracellular stimuli, including tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), lipopolysaccharide, phorbol esters like 12-*O*-tetradecanoylphorbol-13-acetate, and UV irradiation (1–4, 29, 32). In most resting cells, NF-κB is bound to the inhibitory IκB proteins (IκB-α, -β, and -ε) and remains in the cytoplasm as a latent-form transcription factor (1–3, 32). Upon stimulation, IκB becomes phosphorylated on specific serine (Ser) residues (Ser-32 and -36 in IκB-α; Ser-19 and -23 in IκB-β) (1–6, 8). Phosphorylation of IκB triggers its ubiquitination and degradation by the 26S proteosome (29, 31, 33). Proteolysis of IκB proteins releases NF-κB to translocate into the nucleus, where it stimulates transcription of specific target genes (29).

The IκB kinase was first identified as a high-molecular-weight protein complex that can be activated in vitro by MEKK1 or ubiquitination (13). Two subunits of the TNF-α-inducible IκB kinase complex (IKKα and IKKβ, also known as IKK-1 and IKK-2, respectively) that specifically phosphorylate IκB proteins have been recently isolated (9, 21, 25, 28, 34, 38). Activation of the IKKs apparently requires phosphorylation on specific Ser residues (Ser-176 and -180 in IKKα; Ser-177 and -181 in IKKβ), which resemble the consensus MEKK phosphorylation motif (SerXaaXaaXaaSer, where Xaa is any amino acid) (20). One of the upstream effector kinases of the IKKs is NF-κB-inducing kinase (NIK) (19), which is a novel member of the MEKK family and is able to activate both IKKα and IKKβ (25, 34). Through direct interaction with the TNF-α and IL-1 receptor-associated factors (TRAF2, TRAF5, and TRAF6) (19, 25, 27), NIK is thought to mediate the stimulatory effects of TNF-α and IL-1 on the IKK complex. Interestingly, NIK significantly phosphorylates only IKKα on Ser-176, not IKKβ (16). Thus, additional protein kinases may be involved in phosphorylation and activation of the IKKs, especially IKKβ, in response to various extracellular stimuli.

MEKK1 functions as the MAPKKK in the c-Jun NH_2-terminal protein kinase (JNK) signaling pathway (12, 15, 22). MEKK1 phosphorylates and activates JNK-activating kinase (JNKK), which in turn phosphorylates and activates JNK (7, 11, 15, 18, 26, 30, 35). It was suggested that MEKK1 may also participate in regulation of NF-κB activity, since overexpression of MEKK1 induced IκB phosphorylation (13) and NF-κB activation (10, 13). The role of MEKK1 in regulation of the IKK complex, however, is not clear. It was reported previously that a ubiquitination-inducible IκB kinase complex can be activated through phosphorylation by MEKK1 (13). However, it has yet to be determined whether the ubiquitination is required for the TNF-α-induced activation of the IKK complex (9, 21, 25). Although MEKK1 was found to comigrate with the IKK complex during purification of the IKKs (20), there was no evidence that MEKK1 directly interacts with and activates the IKK complex.

Here we report the identification of MEKK1 as an immediate upstream kinase for the IKKs. MEKK1 is activated by TNF-α and IL-1 and potentiates TNF-α-induced NF-κB activation. MEKK1 interacts with and stimulates the activity of the IKKs in cells and directly phosphorylates both IKKα and IKKβ in vitro. Furthermore, we find that MEKK1 acts in parallel with NIK, leading to synergistic activation of the IKK complex. These findings demonstrate that MEKK1 is an immediate upstream kinase of both IKKα and IKKβ and is capable of activating the IKKs in coordination with NIK.

MATERIALS AND METHODS

Cell culture and transfection. HeLa and COS-1 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Transfections were performed as previously described (15, 18).

Plasmids. Hemagglutinin (HA)-IKKβ was constructed by subcloning a PCR-generated *Hin*dIII-*Not*I fragment encoding IKK-2, a gift from Frank Mercurio (Signal Pharmaceuticals, Inc.) (21), into pRc/β-actin expression vector between *Hin*dIII and *Not*I sites, as described elsewhere (9). To construct the mammalian version of glutathione *S*-transferase (GST)–MEKKΔ and the kinase-deficient GST-MEKKΔ (K432M) mutant, an *Nco*I-*Xho*I fragment of MEKKΔ or the

* Corresponding author. Mailing address: Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294. Phone: (205) 975-9225. Fax: (205) 934-1775. E-mail: lin@vh.path.uab.edu.

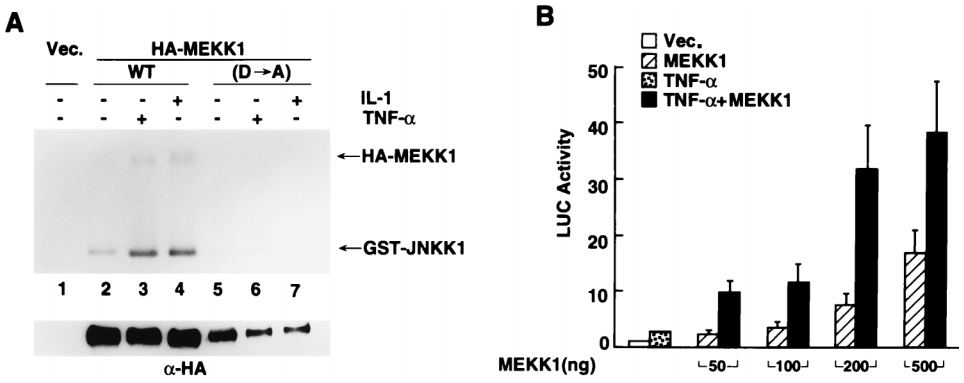

FIG. 1. MEKK1 is activated by TNF-α and IL-1 and potentiates TNF-α-induced NF-κB activation. (A) (Top) COS-1 cells were transfected with expression vectors encoding the full-length HA-MEKK1 or the HA-MEKK1 (D→A) mutant (0.2 μg each) or empty vector, as indicated. After 48 h, cells were treated with TNF-α (50 ng/ml) or IL-1 (10 ng/ml) or left untreated. HA-MEKK1 was isolated by immunoprecipitation with anti-HA monoclonal antibody (12CA5; Santa Cruz). The MEKK1 immunocomplex was incubated at 30°C for 1 h in a kinase buffer (14) containing 10 μCi of 10 μM [γ-^{32}P]ATP with purified bacterial recombinant GST-JNKK1 (2 μg) as a substrate. (Bottom) An aliquot of each lysate was analyzed for its content of MEKK1 or the MEKK1 (D→A) mutant by immunoblotting with anti-HA monoclonal antibody (14). (B) HeLa cells were cotransfected with a 2× NF-κB LUC reporter plasmid (0.1 μg per plate) and expression vector encoding the full-length MEKK1 (50, 100, 200, and 500 ng) or empty vector. After 40 h, the cells were treated with TNF-α (10 ng/ml) for 6 h or left untreated, as indicated. Relative LUC activity was determined as described elsewhere (9, 14). LUC activity expressed by cells transfected with empty vector was given an arbitrary value of 1. The results are presented as means ± standard errors (error bars) and represent two individual experiments. WT, wild type; Vec., vector.

MEKKΔ (K432M) mutant was first subcloned into the pGEX-KG vector. A *Bam*HI-*Cla*I fragment of pGEX-KG MEKKΔ or the MEKKΔ (K432M) mutant was then subcloned into a mammalian GST expression vector between *Bam*HI and *Cla*I sites, as described elsewhere (15). The mammalian GST vectors of IKKs, including IKKα; the IKKα (K44M) mutant, in which the lysine (K) 44 in the ATP binding domain was replaced by methionine (M); the IKKα (AA) mutant, in which Ser-176 and -180 were replaced by alanines; IKKβ; the IKKβ (K44M) mutant, in which the lysine (K) 44 in the ATP binding domain was replaced by methionine (M); and the IKKβ (AA) mutant, in which Ser-177 and -181 were replaced by alanines, were constructed by subcloning PCR-generated *Cla*I-*Not*I fragments of corresponding IKK coding sequences into the mammalian GST expression vector (15). NIK and the kinase-deficient NIK (KK429/430AA) mutant were gifts from David Wallach (The Weizmann Institute of Science).

Purification of recombinant GST fusion proteins. GST-JNKK1; GST–IκB-α (1–54); GST–IκB-α (1–54; TT), in which Ser-32 and -36 were replaced by threonines; and the mammalian versions of GST-MEKKΔ, GST-MEKKΔ (K432M), GST-IKKα, GST-IKKα (K44M), GST-IKKα (AA), GST-IKKβ, GST-IKKβ (K44A), and GST-IKKβ (AA) were purified on glutathione-agarose beads as described elsewhere (8, 9, 15, 18).

Protein kinase assays. Transfected HA-tagged or M2 Flag-IKKs were immunoprecipitated from HeLa or COS-1 cell extracts with anti-HA monoclonal antibody (12CA5; Santa Cruz) or anti-M2 monoclonal antibody (Kodak). The kinase activity of the immune complex was assayed at 30°C for 30 to 60 min in 30 μl of kinase buffer (21) in the presence of 10 μM ATP–10 μCi of [γ-^{32}P]ATP (10 Ci/mmol) with GST–IκB-α or GST–IκB-α (TT) proteins as substrates, as indicated in the figure legends. The reactions were terminated with 4× Laemmli sample buffer. The proteins were resolved by sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis, followed by autoradiography. Radioactivity in the phosphorylated proteins was quantitated by a phosphorimager.

For in vitro phosphorylation of the IKKs by MEKK1, the mammalian version of GST-MEKKΔ, the kinase-deficient mutant GST-MEKKΔ (K432M), wild-type GST-IKKα and GST-IKKβ, the kinase-deficient mutant GST-IKKα (K44M), GST-IKKβ (K44A), GST-IKKα (AA), and GST-IKKβ (AA) were purified to near homogeneity from transfected COS-1 cells. Purified GST-IKK was incubated with or without purified GST-MEKKΔ or the GST-MEKKΔ (K432M) mutant for 1 h in a kinase reaction buffer (15) containing 50 μM ATP–10 μCi of [γ-^{32}P]ATP. Purified bacterial GST-JNKK1, a known substrate for MEKK1, was included as a positive control.

Transcription assays. HeLa cells were cotransfected with a 2× NF-κB luciferase (LUC) reporter plasmid and various expression vectors, as indicated in figure legends. LUC activity was determined as previously described (9, 15).

Immunoprecipitation and immunoblotting analysis. For coimmunoprecipitation of transfected proteins, COS-1 cells were transfected with mammalian expression plasmids encoding various signaling alleles, as indicated in the figure legends. After 30 h, cells were harvested and lysed in lysis buffer (20 mM Tris [pH 7.6], 250 mM NaCl, 3 mM EDTA, 1.5 mM EGTA, 10 mM *p*-nitrophenylphosphate, 1 mM Na_3VO_4, 1% Nonidet P-40, 1 mM dithiothreitol, and 10 mg of aprotinin per ml). After clarification by centrifugation, cell lysates (1 mg) were incubated with anti-HA monoclonal antibody or preimmune serum in the presence of 30 μl (50% [vol/vol]) of protein A-Sepharose beads for 4 h at 4°C. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels, blotted onto Immobilon P membranes (Millipore), and subjected to immunoblotting analysis with specific antibodies as indicated in the figure legends. The antibody-antigen complexes were visualized by the enhanced chemiluminescence detection system (Amersham).

RESULTS

MEKK1 is activated by TNF-α and IL-1 and potentiates TNF-α-induced NF-κB activation. We tested whether MEKK1 is involved in TNF-α or IL-1 signaling pathways that lead to NF-κB activation. COS-1 cells were transiently transfected with expression vectors encoding HA-tagged full-length MEKK1 (HA-MEKK1); HA-MEKK1 (D→A), which is a dominant negative mutant of the full-length MEKK1 (36); or empty expression vector (Fig. 1A). After 48 h, cells were treated with TNF-α or IL-1 or left untreated. HA-MEKK1 was isolated by immunoprecipitation, and its activity was measured in immunocomplex kinase assays with GST-JNKK1, a known substrate of MEKK1 (14). TNF-α and IL-1 stimulated the activity of MEKK1, but not the HA-MEKK1 (D→A) mutant (Fig. 1A). In addition, TNF-α and IL-1 also stimulated the autophosphorylation of MEKK1, which is a characteristic feature of MEKK1 activation (Fig. 1A). These results demonstrate that MEKK1 is part of TNF-α and IL-1 signaling pathways.

We then examined the effect of MEKK1 on TNF-α-induced NF-κB activation. HeLa cells were cotransfected with a 2× NF-κB LUC reporter gene (9), with or without expression vector encoding the full-length MEKK1. After 40 h, the cells were treated with a suboptimal dose of TNF-α for 6 h or left untreated. Treatment with the suboptimal dose of TNF-α induced threefold activation of the 2× NF-κB LUC reporter gene (Fig. 1B). The full-length MEKK1 by itself mildly stimulated the NF-κB reporter gene activity in a dose-dependent manner (Fig. 1B). Coexpression of the full-length MEKK1 enhanced the effect of TNF-α synergistically (Fig. 1B). This result is consistent with previous reports that a dominant negative mutant of MEKK1 was able to block TNF-α-induced NF-κB activation (10, 13), indicating that MEKK1 may be involved in a TNF-α signaling pathway that leads to NF-κB activation.

MEKK1-induced NF-κB activation is mediated by IκB kinases. We and others have shown that MEKK1 may be involved in TNF-α-induced NF-κB activation (Fig. 1) (10, 13).

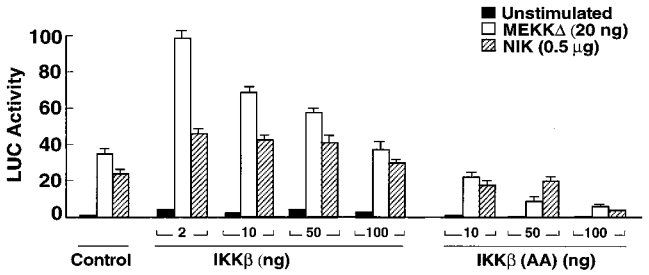

FIG. 2. MEKK1-induced NF-κB activation is mediated by the IKKs. HeLa cells were cotransfected with the 2× NF-κB LUC reporter plasmid (0.5 μg per plate) and expression vectors encoding MEKKΔ (20 ng), NIK (0.5 μg), wild-type IKKβ or the inactive IKKβ (AA) mutant (2, 10, 50, and 100 ng each), or empty vector, as indicated. LUC activity was determined as described for Fig. 1B. The results are presented as means ± standard errors (error bars) and represent three individual experiments.

Since MEKK1 copurified with the IKK complex that controls NF-κB activation in response to extracellular stimuli (21), we determined whether MEKK1-induced NF-κB activation requires the IKKs. HeLa cells were cotransfected with the 2× NF-κB LUC reporter gene, along with expression vectors encoding NIK, or a truncated form of MEKK1, MEKKΔ, which is a specific activator for JNK but not p38 or ERK unless overexpressed (15, 22), in the presence or absence of wild-type IKKβ or the IKKβ (AA) mutant, in which Ser-177 and Ser-181 residues in the putative MEKK1 phosphorylation motif were replaced by alanines (21). Like NIK (34), expression of a small amount of MEKKΔ significantly stimulated NF-κB activation, and the stimulation was potentiated by cotransfected HA-IKKβ and inhibited by the cotransfected HA-IKKβ (AA) mutant in a dose-dependent manner (Fig. 2). The effect of MEKK1 was also modulated by HA-IKKα in a similar manner (24). Thus, MEKK1 activation of NF-κB is mediated by the IKKs.

MEKK1 activates both IKKα and IKKβ in vivo. To determine whether MEKK1 is able to stimulate IKK activity, HeLa cells were transiently transfected with expression vectors encoding HA-IKKα or HA-IKKβ, with or without NIK or MEKKΔ. After 48 h, the cells were treated with TNF-α or left untreated. HA-IKK was isolated by immunoprecipitation, and its activity was measured by immunocomplex kinase assays with GST–IκB-α or the GST–IκB-α (TT) mutant as a substrate (3). The GST–IκB-α (TT) mutant is a very poor IKK substrate because Ser-32 and Ser-36 residues were replaced by threonine residues (5, 6, 8). Like TNF-α (Fig. 3A, lanes 8 and 16) (9, 21, 34, 38) and NIK (Fig. 3A, lanes 7 and 15) (25, 34), expression of a small amount of MEKKΔ significantly stimulated the activities of both HA-IKKα and HA-IKKβ (Fig. 3A, lanes 6 and 14). The activation of the IKKs is specific since they phosphorylated only GST–IκB-α, not the GST–IκB-α (TT) mutant (9) (Fig. 3A, lanes 10 and 18). This activation was not a result of increased expression of the HA-IKKs, as demonstrated by immunoblotting analysis (Fig. 3A). Under the same conditions, the HA-IKK (AA) mutants (21) were not activated by cotransfected MEKKΔ, NIK, or TNF-α treatment (24). Expression of the full-length MEKK1 mildly stimulated IKKβ activity in a

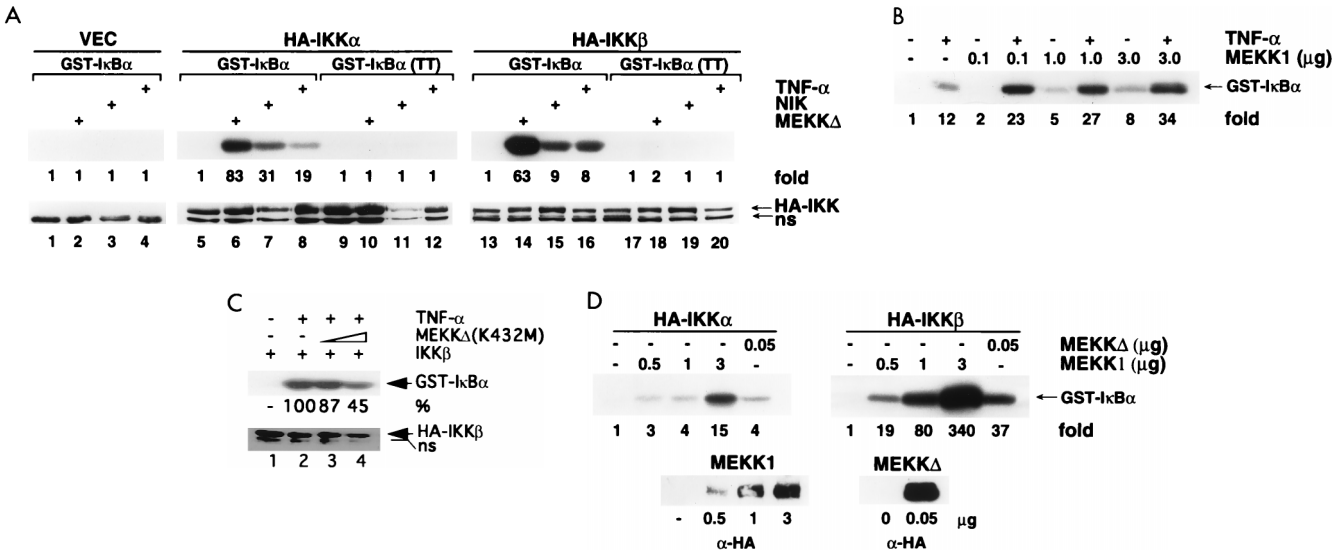

FIG. 3. Activation of the IKKs and NF-κB by MEKK1 in vivo. (A) Activation of IKKα and IKKβ by cotransfected MEKKΔ, NIK, or TNF-α treatment. (Top) HeLa cells were transfected with expression vectors encoding HA-IKKα or HA-IKKβ (3 μg per plate each), in the presence or absence of MEKKΔ (0.1 μg), NIK (3 μg), or empty vector, as indicated. After 48 h, the cells were either treated with TNF-α (50 ng/ml) for 10 min or left untreated. HA-IKK was immunoprecipitated, and its activity was determined by immunocomplex kinase assays with GST–IκB-α or GST–IκB-α (TT) as a substrate, as described elsewhere (9). Substrate phosphorylation was quantitated with a phosphorimager. Fold stimulation is indicated. (Bottom) An aliquot of each lysate was analyzed for its content of IKK by immunoblotting (14). (B) Coexpression of the full-length MEKK1 potentiates TNF-α-induced IKKβ activation. HeLa cells were transfected with expression vectors encoding HA-IKKβ (0.1 μg) in the presence or absence of the full-length MEKK1 (0.1, 1, and 3 μg) as indicated. After 48 h, cells were treated with TNF-α (100 ng/ml) for 10 min or left untreated, as indicated. HA-IKKβ was immunoprecipitated, and its activity was determined as described for panel A. (C) Inhibition of TNF-α-induced IKKβ activation by the dominant negative mutant of MEKK1. (Top) HeLa cells were transfected with expression vectors encoding HA-IKKβ (3 μg) with or without the dominant negative form of MEKK1 (lane 3, 1 μg, and lane 4, 2 μg). After 40 h, the cells were treated with TNF-α (50 ng/ml) for 5 min or left untreated, as indicated. HA-IKKβ was immunoprecipitated, and its activity was determined as described for panel A. (Bottom) An aliquot (30 μg) of each sample was analyzed by immunoblotting analysis with anti-HA antibody for its content of IKKβ and used to normalize the IKKβ activity. (D) Comparison of activation of IKKα and IKKβ by the full-length MEKK1 to that by MEKKΔ. (Top) COS-1 cells were transfected with expression vectors encoding HA-IKKα (2 μg) or HA-IKKβ (0.1 μg), in the presence or absence of either the full-length MEKK1 (0.5, 1, and 3 μg) or MEKKΔ (0.05 μg), or empty vector, as indicated. After 48 h, the cells were harvested. HA-IKK was immunoprecipitated, and its activity was determined as described for panel A. (Bottom) An aliquot of each lysate was analyzed for its content of MEKK1 and MEKKΔ by immunoblotting with anti-HA monoclonal antibody (14). VEC, vector; ns, nonspecific.

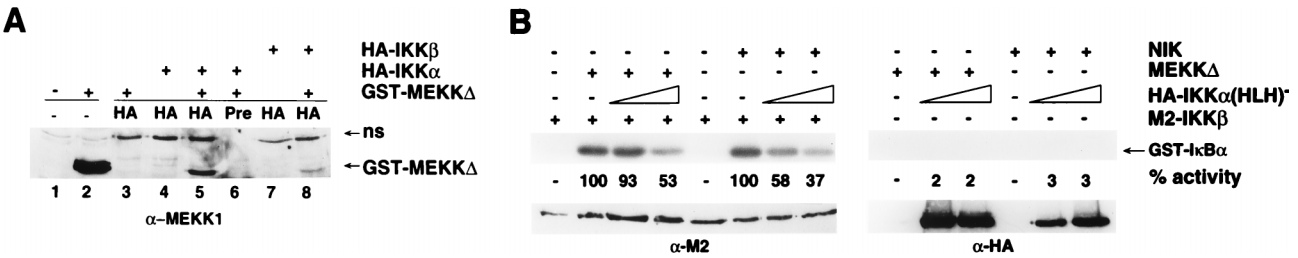

FIG. 4. (A) MEKK1 interacts with the IKKs in vivo. COS-1 cells were cotransfected with expression vectors encoding mammalian GST-MEKKΔ (0.2 μg per plate), HA-IKKα, HA-IKKβ (3 μg each), or empty vector, as indicated. After 24 to 30 h, cells were harvested. The lysates were immunoprecipitated with anti-HA monoclonal antibody (HA; lanes 3, 4, 5, 7, and 8) or control antibody (Pre; lane 6) and then analyzed by immunoblotting with an antibody against the C-terminal region of MEKK1 (C-22; Santa Cruz). Lysates of empty vector and GST-MEKKΔ-transfected cells were also directly analyzed by immunoblotting (lanes 1 and 2). (B) The IKKα $(HLH)^-$ mutant inhibits IKK activation by MEKK1. (Top) COS-1 cells were cotransfected with expression vectors encoding M2-IKKβ (0.1 μg per plate), along with MEKKΔ (20 ng), NIK (1 μg), or empty vector, in the presence or absence of the HA-IKKα $(HLH)^-$ mutant (lanes 3 and 7, 1 μg each; lanes 4 and 8, 2 μg each; lanes 10 and 13, 0.5 μg each; lanes 11 and 14, 1 μg each), as indicated. After 48 h, the cells were harvested. M2-IKKβ and HA-IKKα $(HLH)^-$ mutant were immunoprecipitated, and their activities were determined, respectively, as described elsewhere (9). (Bottom) An aliquot of each lysate was analyzed for its content of IKK by immunoblotting with anti-M2 (left panel) or anti-HA monoclonal antibody (right panel), as indicated elsewhere (14). ns, nonspecific.

dose-dependent manner and potentiated the stimulatory effect of TNF-α on IKKβ activity (Fig. 3B). Conversely, expression of a dominant negative form of MEKK1, MEKKΔ (K432M), partially blocked activation of IKKβ by TNF-α (Fig. 3C). In COS-1 cells, expression of the full-length MEKK1 also stimulated the activities of both IKKα and IKKβ in a dose-dependent manner (Fig. 3D). In comparison to MEKKΔ, however, the full-length MEKK1 was less potent (Fig. 3B and D). These results indicate that MEKK1 may act as an upstream activator for both IKKα and IKKβ in response to extracellular stimuli such as TNF-α.

MEKK1 interacts with the IKKs in vivo. Next we examined the interaction between MEKK1 and the IKKs. COS-1 cells were cotransfected with expression vectors encoding HA-IKKα or HA-IKKβ, with or without the mammalian version of GST-MEKKΔ. After 30 h, cells were harvested and the lysates were immunoprecipitated with anti-HA monoclonal antibody (12CA5; Santa Cruz) or control antibody. Immunoblotting analysis with an antibody against the C-terminal region of MEKK1 (C-22; Santa Cruz) revealed that GST-MEKKΔ was specifically coimmunoprecipitated with both HA-IKKα (Fig. 4A, lane 5) and HA-IKKβ (Fig. 4A, lane 8), with similar affinities. These results demonstrate that MEKK1 may physically interact with the IKKs in vivo.

The C-terminal helix-loop-helix (HLH) domain of IKK has been proposed to be involved in interaction with the upstream regulators of IKKs (25, 36), and mutations that disrupt the HLH motif resulted in greatly reduced IKK activity in response to TNF-α stimulation or overexpression of IKKβ (38). Therefore, we examined whether a mutant with the defective HLH motif could affect IKK activation by MEKK1. Coexpression of the HA-IKKα $(HLH)^-$ mutant (38), in which leucine 605 and phenylalanine 606 were replaced with arginine and proline, respectively, resulted in inhibition of M2-IKKβ activation by cotransfected MEKKΔ (50%) or NIK (60%) in a dose-dependent manner (Fig. 4B, left panel). The inhibition was not a result of changes in expression of M2-IKKβ, as demonstrated by immunoblotting analysis (Fig. 4B). The HA-IKKα $(HLH)^-$ mutant itself had very low activity (Fig. 4B, right panel), as previously reported (38). In addition, the untagged IKKα $(HLH)^-$ mutant inhibited the activation of HA-IKKα by MEKKΔ and NIK, although to a lesser extent (24). Thus, an intact HLH domain activation may be required for activation of the IKKs by MEKK1, as it was for activation by NIK (25).

MEKK1 is an immediate upstream kinase for the IKKs. To determine whether MEKK1 activates IKK directly, we tested the ability of MEKK1 to phosphorylate IKK in in vitro kinase assays. Because bacterial GST-IKK was insoluble (24), we constructed mammalian GST versions of the kinase-deficient IKKα (K44M) and IKKβ (K44A) mutants, in which lysine 44 residues in the ATP binding sites were replaced with methionine or alanine (9, 21). Mammalian GST-MEKKΔ and its kinase-deficient mutant GST-MEKKΔ (K432M) were also constructed. The fusion proteins were expressed and purified from COS-1 cells to near homogeneity (Fig. 5A). GST-JNKK1, the known MEKK1 substrate (15), was used as a positive control. Purified GST-MEKKΔ significantly phosphorylated both GST-IKKα (K44M) and GST-IKKβ (K44A) (Fig. 5B, lanes 2 and 5), as well as GST-JNKK1 (Fig. 5B, lane 8). In contrast, the kinase-deficient mutant GST-MEKKΔ (K432M) failed to phosphorylate GST-IKKα (K44M), GST-IKKβ (K44A), or GST-JNKK1 (Fig. 5B, lanes 3, 6, and 9). Under the same conditions, phosphorylation of the GST-IKK (AA) mutants by GST-MEKKΔ was greatly reduced in comparison to phosphorylation of wild-type GST-IKKs (24). These data indicate that MEKK1 may phosphorylate the IKKs directly, probably on the serine residues in the putative MEKK1 phosphorylation motif. Although we cannot formally rule out the possibility that the phosphorylation of GST-IKK might be carried out by an unknown protein which copurified with GST-MEKKΔ, this does not appear likely, since no other proteins were detected in the preparation of purified GST-MEKKΔ (Fig. 5A, lane 3).

Synergistic activation of IKKβ by MEKK1 and NIK. Because both NIK and MEKK1 activate IKK, we examined the relationship between NIK and MEKK1 in respect to IKK activation. In HeLa cells, expression of wild-type NIK was able to stimulate HA-IKKβ activity in a dose-dependent manner (Fig. 6A, lanes 2 and 3), and expression of a small amount of MEKKΔ alone also activated HA-IKKβ (Fig. 6A, lane 4). Coexpression of MEKKΔ and NIK together enhanced NIK-stimulated IKKβ activity synergistically (Fig. 6A, lanes 5 and 6). Consistently, the effect of NIK on NF-κB activation was also potentiated by MEKKΔ. In transcription assays, expression of a suboptimal amount of wild-type NIK stimulated the activity of NF-κB in a dose-dependent manner, as measured by the 2× NF-κB LUC reporter gene (Fig. 6B, thick-diagonal-stripe bars). Expression of a small amount of MEKKΔ mildly stimulated NF-κB activation (Fig. 6B, open bar). Coexpression of MEKKΔ and NIK, however, synergistically stimulated NF-κB activation (Fig. 6B, thin-diagonal-stripe bars). In reciprocal experiments, coexpression of NIK also augmented MEKKΔ-induced IKKβ activity (Fig. 6C) and NF-κB activation synergistically (Fig. 6D).

The synergistic activation of IKKβ by MEKK1 and NIK does

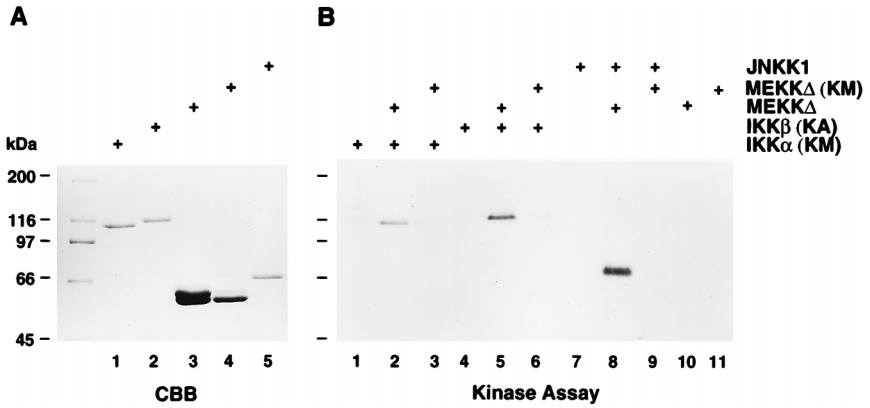

FIG. 5. Phosphorylation of the IKKs by MEKKΔ in vitro. (A) Mammalian versions of the GST-IKKα (K44M) mutant (0.4 μg), the GST-IKKβ (K44A) mutant (0.4 μg), GST-MEKKΔ (3 μg), and the kinase-deficient GST-MEKKΔ (K432M) mutant (1 μg) were purified from COS-1 cells to near homogeneity. GST-JNKK1 (0.4 μg) was also purified from bacteria. The purified fusion proteins were analyzed by SDS–9% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue (CBB). (B) Purified mammalian GST-IKK kinase-deficient mutants (0.2 μg each) were incubated with or without purified MEKKΔ (0.1 μg) or the MEKKΔ (K432M) mutant (0.1 μg) for 1 h in a kinase buffer (14) containing 50 μM ATP–10 μCi of [γ-^{32}P]ATP. Purified bacterial GST-JNKK1 (0.4 μg) (14) was included as a positive control. This experiment was repeated three times with similar results.

not reveal whether NIK and MEKK1 act sequentially, or in parallel, to activate IKKβ. Therefore, we examined the effect of a dominant negative MEKK1 mutant, MEKKΔ (K432M) (22), on the activation of IKKβ by NIK. Expression of the MEKKΔ (K432M) mutant resulted in at least 70% inhibition of HA-IKKβ activation by cotransfected NIK in a dose-dependent manner (Fig. 7A). Consistently, expression of the MEKKΔ (K432M) mutant also abolished NIK-induced NF-κB activation in transcription assays, as measured by the activity of the 2× NF-κB LUC reporter gene (Fig. 7B). In reciprocal experiments, expression of a dominant negative NIK mutant, NIK (KK429/430AA), in which the lysine residues in the ATP binding site were replaced with alanines (8), mildly inhibited HA-IKKβ activation by MEKKΔ (Fig. 7C) and partially blocked MEKKΔ-induced NF-κB activation (Fig. 7D). These results suggest that MEKK1 and NIK may act in parallel to stimulate IKK activity. The mutual inhibitions exerted by the dominant negative mutants of MEKK1 and NIK indicate that MEKK1 and NIK might have a common docking region on the IKKs (25, 36).

DISCUSSION

In this report, we demonstrate that MEKK1 may play an important role in regulation of the IκB kinase complex and NF-κB activation in response to extracellular stimuli. This con-

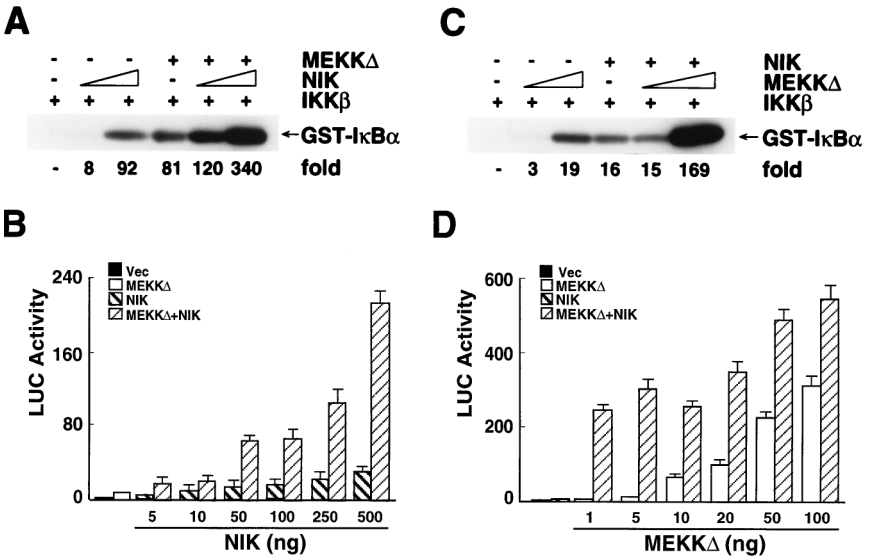

FIG. 6. Synergistic activation of IKKβ and NF-κB by cotransfected MEKKΔ and NIK expression vectors. (A) HeLa cells were cotransfected with HA-IKKβ (3 μg per plate) with or without MEKKΔ (20 ng) and NIK (3 and 5 μg). The activity of HA-IKKβ was determined as described for Fig. 3A. (B) HeLa cells were cotransfected with the 2× NF-κB LUC reporter plasmid (0.5 μg per plate) and expression vectors encoding MEKKΔ (20 ng) in the presence or absence of NIK (5, 10, 50, 100, 250, and 500 ng). LUC activity was determined as described for Fig. 1B. This experiment was repeated three times with similar results. (C) HeLa cells were cotransfected with expression vectors encoding HA-IKKβ (3 μg per plate) with or without NIK (4 μg) and MEKKΔ (10 and 50 ng). The activity of HA-IKKβ was determined as described for Fig. 3A. (D) HeLa cells were cotransfected with the 2× NF-κB LUC reporter plasmid (0.5 μg per plate) and expression vectors encoding NIK (0.25 μg) with or without MEKKΔ (1, 5, 10, 20, 50, and 100 ng). LUC activity was determined as described for Fig. 1B. This experiment was repeated three times with similar results. Vec, vector.

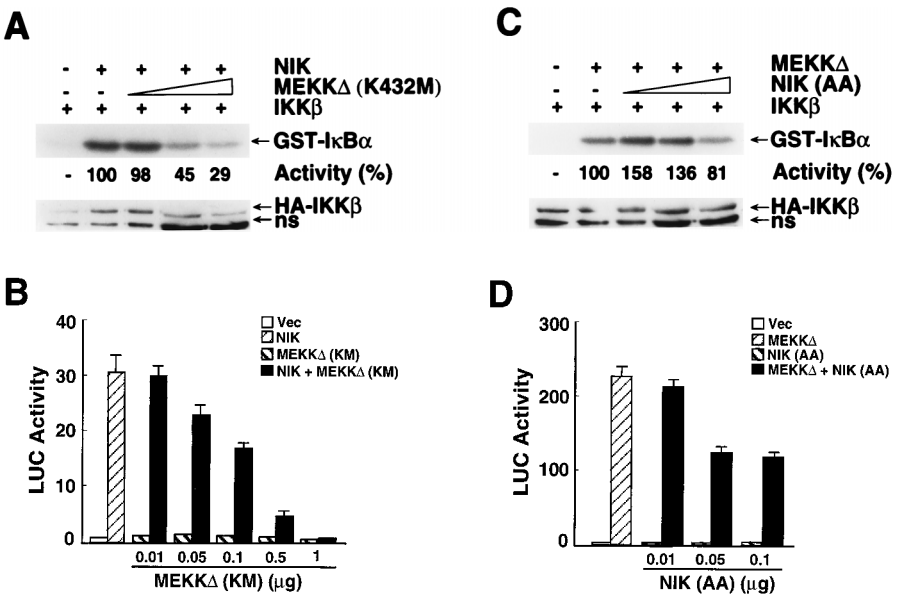

FIG. 7. MEKKΔ and NIK act in parallel to stimulate IKKβ and NF-κB activity. (A) (Top) HeLa cells were cotransfected with HA-IKKβ (3 μg per plate) with or without NIK (3 μg) and MEKKΔ (K432M) (1, 2, and 4 μg). HA-IKKβ was immunoprecipitated from cell extracts that had been normalized to contain equal or greater amounts of IKKβ proteins compared to NIK alone (lanes 1, 2, and 3, 30 μg each; lanes 4 and 5, 150 μg each). The activity of HA-IKKβ was determined as described for Fig. 3A. (Bottom) An aliquot of each sample (lanes 1, 2, and 3, 15 μg each; lanes 4 and 5, 75 μg each) was immunoblotted for its content of IKKβ. (B) HeLa cells were cotransfected with the 2× NF-κB LUC reporter plasmid (0.5 μg per plate) and expression vectors encoding NIK (0.5 μg) with or without MEKKΔ (K432M) (0.01, 0.05, 0.1, 0.5, and 1 μg). LUC activity was determined as described for Fig. 1B. This experiment was repeated three times with similar results. (C) HeLa cells were cotransfected with expression vectors encoding HA-IKKβ (3 μg per plate) with or without MEKKΔ (0.1 μg) and NIK (KK428-430AA) (1, 2, and 3 μg). HA-IKKβ was immunoprecipitated from cell extracts that had been normalized to its content of IKKβ proteins (lanes 1, 2, and 3, 30 μg each; lanes 4 and 5, 150 μg each). The activity of HA-IKKβ was determined as described for Fig. 3A. (Bottom) An aliquot of each sample (lanes 1, 2, and 3, 15 μg each; lanes 4 and 5, 75 μg each) was immunoblotted for its content of IKK. (D) HeLa cells were cotransfected with the 2× NF-κB LUC reporter plasmid (0.5 μg per plate) and expression vectors encoding MEKKΔ (50 ng) with or without NIK (KK428-430AA) (10, 50, and 100 ng). LUC activity was determined as described for Fig. 1B. This experiment was repeated three times with similar results. ns, nonspecific; Vec, vector.

clusion is based on several lines of evidence. First, TNF-α and IL-1, two potent extracellular signals that stimulate IKK activity and NF-κB activation, stimulated MEKK1 activity (Fig. 1A), and the effect of TNF-α on NF-κB activation was potentiated by MEKK1 (Fig. 1B). Second, expression of MEKK1 by itself stimulated NF-κB activation, and its effect was mediated by IKKβ (Fig. 2). These results are consistent with previous reports that a dominant negative mutant of MEKK1 was able to block TNF-α-induced NF-κB activation (10, 13) and suggest that MEKK1 may be part of the TNF-α signaling pathway that leads to NF-κB activation. Third, expression of MEKK1 or MEKKΔ, the activated form of MEKK1, stimulated the activities of both IKKα and IKKβ in transfected cells (Fig. 3A) and potentiated the effect of TNF-α on IKKβ activation (Fig. 3B). Conversely, the dominant negative form of MEKK1 partially blocked the activation of IKKβ by TNF-α (Fig. 3C). Finally, MEKK1 physically interacted with the IKKs in vivo and directly phosphorylated the IKKs in vitro (Fig. 4A and 5B), suggesting that MEKK1 may be an immediate upstream kinase for IKKs. After submission of the manuscript, Gaynor and his colleagues reported that Tax, a viral protein of human T-cell leukemia virus type 1, binds to and activates MEKK1, resulting in stimulation of IKK activity and NF-κB activation (37). In addition, it was recently reported by Maniatis and his colleagues that MEKK1 was able to activate both IKKα and IKKβ and induced phosphorylation of IKKs in the IKK complex (14). These findings further support our conclusion and suggest that MEKK1 may play a critical role in NF-κB activation through stimulation of the IKKs in response to extracellular stimuli such as human T-cell leukemia virus type 1 and TNF-α.

The full-length MEKK1 and MEKKΔ stimulated the activities of two catalytic subunits of the IKK complex, IKKα and IKKβ, in vivo. The putative MEKK1 phosphorylation motif appears to be required for activation of the IKKs by MEKK1 (24), as it does for NIK or TNF-α treatment (21, 25). In COS-1 cells, both the full-length MEKK1 and MEKKΔ activated IKKβ more effectively than IKKα (Fig. 3D), consistent with recent reports that MEKK1 may activate IKKβ differentially (23, 37). However, this apparent difference in activation does not necessarily exclude the possibility that MEKK1 may still be an upstream activator of IKKα. IKKα has less intrinsic activity than IKKβ in several cell lines examined, including COS-1 and 293 cells (Fig. 3) (38). A larger amount of expression vector encoding IKKα needs to be transfected into the cells in order to generate considerable activity. This results in a higher basal level of IKKα activity and a lesser degree of its activation by the full-length MEKK1 or MEKKΔ. This is consistent with an earlier report that, with increased amounts of transfected IKKs, the fold stimulation by TNF-α was decreased (38). Interestingly, we found that both IKKα and IKKβ were activated by MEKKΔ to a similar extent in HeLa cells (Fig. 3A). One possible explanation is that expression levels of the IKKs are much lower in HeLa cells than they are in COS-1 cells (24). Consequently, the basal activities of the IKKs were much lower and can be stimulated to a greater extent by MEKKΔ.

The full-length MEKK1 and its activated form MEKKΔ may respond differently to extracellular stimuli, since MEKKΔ lacks the N-terminal domain that is presumably required for interaction with its regulators (36, 37). However, MEKKΔ can still act as a specific activator in transfection experiments, since it activates only JNK, and not p38 or ERK unless overexpressed (15, 22). In this report, we found that the full-length

MEKK1 and MEKKΔ behaved in a similar manner in respect to IKK activation in transfection assays where the amount of MEKKΔ was kept very low. For example, both full-length MEKK1 and MEKKΔ are apparently better activators for IKKβ than for IKKα in transfected COS-1 cells (Fig. 3D).

Activation of the IKKs by MEKK1 is likely due to direct phosphorylation. Purified mammalian GST-MEKKΔ, but not its kinase-deficient mutant GST-MEKKΔ (K432M), significantly phosphorylated both GST-IKKα and GST-IKKβ in vitro (Fig. 5B). Under the same conditions, phosphorylation of the IKK (AA) mutants by MEKKΔ was greatly reduced (24). This indicates that the serine residues in the putative MEKK1 phosphorylation motif may be the major phosphorylation acceptors used by MEKK1. However, it was reported that the IKKβ (S177A) mutant, in which Ser-177 was replaced by alanine, had the same basal activity as its wild-type counterpart (16). It will be of interest to determine whether both of the serines in the MEKK1 phosphorylation motif are indeed phosphorylated and required for activation by MEKK1. We have also found that autophosphorylation of the IKK (AA) mutants was severely impaired (24). One possibility is that phosphorylation by MEKK1 or a MEKK1-like kinase is required for the IKKs to undergo productive autophosphorylation. Another possibility is that one of the putative MEKK1 phosphorylation sites may be the same site as that for IKK autophosphorylation. Further studies are needed to determine the exact site(s) and the effect of autophosphorylation on the activities of the IKKs.

The function of MEKK1 in TNF-α-induced NF-κB activation has been controversial (10, 13, 17, 27). Recent studies (14, 23, 37) and the results presented here suggest that MEKK1 may contribute to NF-κB activation induced by TNF-α and IL-1, apart from its critical role in mediating Tax-induced NF-κB activation (37). On the other hand, NIK may also play an important role in mediating TNF-α-induced NF-κB activation (25). The role of NIK in mediating Tax-induced NF-κB activation has yet to be determined.

MEKK1 physically interacts with the IKKs in vivo (Fig. 4A), and the interaction may involve a docking region that is shared by NIK. This could explain why wild-type NIK and MEKK1 could activate the IKKs synergistically (Fig. 6) but have their effects be blocked by each other's interfering mutants (Fig. 7). Wild-type NIK and MEKK1 can phosphorylate and then dissociate from the IKKs; this would allow the other kinase to bind to and further activate the IKKs, leading to synergistic activation (Fig. 6A and C). Conversely, the dominant negative mutants of NIK and MEKKΔ, which are catalytically inactive, would occupy such a docking region on the IKKs and remain there for a much longer period of time. This would prevent the other kinase from binding to and phosphorylating the IKKs, resulting in inhibition (Fig. 7A and C). Furthermore, the MEKKΔ (K432M) mutant has a much more pronounced inhibitory effect on activation of IKK and NF-κB by NIK than does the dominant negative NIK (AA) mutant on MEKKΔ-stimulated IKK and NF-κB activation (Fig. 7). The simplest explanation is that MEKK1 may interact with both IKKs and productively activate the IKK complex while NIK preferentially interacts with IKKα (16, 25, 34). Therefore, even in the presence of excess wild-type NIK, the MEKKΔ (K432M) mutant would still have a concentration advantage in the microenvironment at the IKKs' docking region because it interacts with both IKKs, resulting in inhibition of NIK activation (Fig. 7A and B).

In comparison to the MEKK1 (K432M) mutant, the dominant negative NIK (AA) mutant appears to be a less potent inhibitor of IKK activation by MEKKΔ (Fig. 7C and D). The partial inhibition by the NIK (AA) mutant suggests that NIK might act upstream of MEKK1. However, we were unable to detect activation of MEKK1 by cotransfection of NIK (24). It is more likely that the NIK (AA) mutant might occupy only the docking region of IKKα, allowing MEKKΔ to interact with the docking region of IKKβ and position itself to act on IKKβ and then IKKα once the NIK (AA) mutant dissociates. This scenario is further supported by the observations that NIK preferentially interacts with IKKα rather than IKKβ (25, 34). The fact that the IKKα $(HLH)^-$ mutant was able to inhibit IKK activation by MEKK1 and NIK (Fig. 5) supports the notion that the HLH domain may be required for IKK activation by its upstream activators (38). Whether the HLH domain is, however, part of the docking region overlapped between MEKK1 and NIK has yet to be determined. Further mutational analysis of IKK is needed to map the binding region(s) in the IKKs that is involved in their interaction with both MEKK1 and NIK. Investigation of coordinate regulation by MEKK1 and NIK should provide new insights into how specificity and diversity are achieved for the signaling pathways that lead to activation of the IKK complex and NF-κB.

ACKNOWLEDGMENTS

We thank M. Karin, F. Mercurio, Melanie H. Cobb, G. L. Johnson, and D. Wallach for the different plasmids that made this work possible and F. Mercurio for helpful discussions.

This work was supported by National Institutes of Health grant CA73740 and American Heart Association Scientist Development grant 9630261N (A.L.).

REFERENCES

1. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
2. **Baeuerle, P. A., and D. Baltimore.** 1996. NF-κB: ten years after. Cell **87:**13–20.
3. **Baldwin, A. S.** 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. **14:**649–683.
4. **Barnes, P. J., and M. Karin.** 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. N. Engl. J. Med. **336:**1066–1071.
5. **Brockman, J. A., D. C. Scherer, T. A. McKinsey, S. M. Hall, X. Qi, W. Y. Lee, and D. W. Ballard.** 1995. Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. Mol. Cell. Biol. **15:**2809–2818.
6. **Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist.** 1995. Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science **267:**1485–1488.
7. **Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and R. J. Davis.** 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267:**682–685.
8. **DiDonato, J. A., F. Mercurio, C. Rosette, J. Wu-Li, H. Suyang, S. Ghosh, and M. Karin.** 1996. Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol. Cell. Biol. **16:**1295–1304.
9. **DiDonato, J. A., M. Hayakawa, D. M. Rothwarf, E. Zandi, and M. Karin.** 1997. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature **388:**548–554.
10. **Hirano, M., S. Osada, T. Aoki, S. Hirai, M. Hosaka, J. Inoue, and S. Ohno.** 1996. MEK kinase is involved in tumor necrosis factor alpha-induced NF-κB activation and degradation of IκB-α. J. Biol. Chem. **271:**13234–13238.
11. **Holland, P. M., M. Suzanne, J. S. Campbell, S. Noselli, and J. A. Cooper.** 1997. MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. J. Biol. Chem. **272:**24994–24998.
12. **Lange-Carter, C. A., C. M. Pleiman, A. M. Gardner, K. J. Blumer, and G. L. Johnson.** 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science **260:**315–319.
13. **Lee, F. S., J. Hagler, Z. J. Chen, and T. Maniatis.** 1997. Activation of the IκBα kinase complex by MEKK1, a kinase of the JNK pathway. Cell **88:**213–222.
14. **Lee, F. S., R. T. Peters, L. C. Dang, and T. Maniatis.** 1998. MEKK1 activates both IκB kinase a and IκB kinase b. Proc. Natl. Acad. Sci. USA **95:**9319–9324.
15. **Lin, A., A. Minden, H. Martinetto, F. X. Claret, C. Lange-Carter, F. Mercurio, G. L. Johnson, and M. Karin.** 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science **268:**286–290.
16. **Ling, L., Z. Cao, and D. V. Goeddel.** 1998. NF-κB-inducing kinase activates IKK-α by phosphorylation of Ser-176. Proc. Natl. Acad. Sci. USA **95:**3792–3797.
17. **Liu, Z. G., H. Hsu, D. V. Goeddel, and M. Karin.** 1996. Dissection of TNF

receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell **87:**565–576.
18. **Lu, X., S. Nemoto, and A. Lin.** 1997. Identification of c-Jun NH_2-terminal protein kinase (JNK)-activating kinase 2 as an activator of JNK but not p38. J. Biol. Chem. **272:**24751–24754.
19. **Malinin, N. L., M. P. Boldin, A. V. Kovalenko, and D. Wallach.** 1997. MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. Nature **385:**540–544.
20. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Wound, and N. G. Ahn.** 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:** 966–970.
21. **Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. F. Rao.** 1997. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science **278:**860–866.
22. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
23. **Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura.** 1998. Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase 1. Proc. Natl. Acad. Sci. USA **95:**3537–3542.
24. **Nemoto, S., and A. Lin.** Unpublished results.
25. **Regnier, C. H., H. Y. Song, X. Gao, D. V. Goeddel, Z. Cao, and M. Rothe.** 1997. Identification and characterization of an IκB kinase. Cell **90:**373–383.
26. **Sanchez, I., R. T. Hughes, B. J. Mayer, K. Yee, J. R. Woodgett, J. Avruch, J. M. Kyriakis, and L. I. Zon.** 1994. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature **372:** 794–798.
27. **Song, H. Y., C. H. Regnier, C. J. Kirschning, T. M. Ayres, D. V. Goeddel, and M. Rothe.** 1997. TNF-mediated kinase cascades: bifurcation of NF-κB and JNK/SAPK pathways at TRAF2. Proc. Natl. Acad. Sci. USA **94:**9792–9796.
28. **Stancovski, I., and D. Baltimore.** 1997. NF-κB activation: the IκB kinase revealed? Cell **91:**299–302.
29. **Thanos, D., and T. Maniatis.** 1995. NF-κB: a lesson in family values. Cell **80:** 529–532.
30. **Tournier, C., A. J. Whitmarsh, J. Cavanagh, T. Barrett, and R. J. Davis.** 1997. Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH_2-terminal kinase. Proc. Natl. Acad. Sci. USA **94:**7337–7342.
31. **Traenckner, E. B., S. Wilk, and P. A. Baeuerle.** 1994. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκBα that is still bound to NF-κB. EMBO J. **13:**5433–5441.
32. **Verma, I. M., J. K. Stevenson, E. M. Schwarz, D. Van Antwerp, and S. Miyamoto.** 1995. Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes Dev. **9:**2723–2735.
33. **Whiteside, T., M. K. Ernst, O. LeBail, C. Laurent-Winter, N. Rice, and A. Israel.** 1995. N- and C-terminal sequences control degradation of MAD3/IκBα in response to inducers of NF-κB activity. Mol. Cell. Biol. **15:**5339–5345.
34. **Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel.** 1997. IκB kinase-β: NF-κB activation and complex formation with IκB kinase α and NIK. Science **278:**866–869.
35. **Wu, Z., J. Wu, E. Jacinto, and M. Karin.** 1997. Molecular cloning and characterization of human JNKK2, a novel Jun NH_2-terminal kinase-specific kinase. Mol. Cell. Biol. **17:**7407–7416.
36. **Xu, S., D. J. Robbins, L. B. Christerson, J. M. English, C. A. Vanderbilt, and M. H. Cobb.** 1996. Cloning of rat MEK kinase 1 cDNA reveals an endogenous membrane-associated 195-kDa protein with a large regulatory domain. Proc. Natl. Acad. Sci. USA **93:**5291–5295.
37. **Yin, M.-J., L. B. Christerson, Y. Yamamoto, Y.-T. Kwak, S. Xu, F. Mercurio, M. Barbosa, M. H. Cobb, and R. B. Gaynor.** 1998. HTLV-1 Tax protein binds to MEKK1 to stimulate IκB kinase activity and NF-κB activation. Cell **93:** 875–884.
38. **Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin.** 1997. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. Cell **91:** 243–252.