Mitogen-activated Protein Kinase/ERK Kinase Kinases 2 and 3 Activate Nuclear Factor-κB through IκB Kinase-α and IκB Kinase-β*

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Recent evidence indicates that nuclear factor-κB (NF-κB), a transcription factor critically important for immune and inflammatory responses, is activated by a protein kinase cascade. The essential features of this cascade are that a mitogen-activated protein kinase kinase kinase (MAP3K) activates an IκB kinase (IKK) that site-specifically phosphorylates IκB. The IκB protein, which ordinarily sequesters NF-κB in the cytoplasm, is subsequently degraded by the ubiquitin-proteasome pathway, thereby allowing the nuclear translocation of NF-κB. Thus far, only two MAP3Ks, NIK and MEKK1, have been identified that can activate this pathway. We now show that MEKK2 and MEKK3 can in vivo activate IKK-α and IKK-β, induce site-specific IκBα phosphorylation, and, relatively modestly, activate an NF-κB reporter gene. In addition, dominant negative versions of either IKK-α or IKK-β abolish NF-κB activation induced by MEKK2 or MEKK3, thereby providing evidence that these IKKs mediate the NF-κB-inducing activities of these MEKKs. In contrast, other MAP3Ks, including MEKK4, ASK1, and MLK3, fail to show evidence of activation of the NF-κB pathway. We conclude that a distinct subset of MAP3Ks can activate NF-κB.

The transcription factor nuclear factor-κB (NF-κB) plays a critical role in immune and inflammatory responses (1, 2). NF-κB, prototypically a heterodimer of p50 and p65 subunits, is sequestered in the cytoplasm of most cell types by virtue of its association with a family of inhibitor molecules, the IκBs.

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† The abbreviations used are: NF-κB, nuclear factor-κB; ASK1, apoptosis signal-regulating kinase 1; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; HTTLV-I, human T-cell leukemia virus, type I; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP2K, mitogen-activated protein kinase kinase; MAP3K, mitogen-activated protein kinase kinase kinase; MEKK, mitogen-activated protein kinase/ERK kinase; MLK3, mixed-lineage kinase 3; NIK, NF-κB inducing kinase; PAGE, polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor α.

Upon exposure to a wide variety of agents, including the proinflammatory cytokine TNF-α, lipopolysaccharide, oxidative stress, and the HTTLV-I Tax protein, the IκB protein is phosphorylated at its N terminus. In the case of IκBα, the most extensively studied IκB isoform, this phosphorylation occurs at Ser-32 and Ser-36 (3, 4). This phosphorylation event targets IκB for degradation by the ubiquitin-proteasome pathway (5), allowing the subsequent nuclear translocation of NF-κB.

An IκB kinase (IKK) complex with a native molecular mass of 700 kDa was originally identified in cytoplasmic extracts of HeLa cells and shown to perform the site-specific phosphorylation of IκBα (6, 7). A significant advance was the subsequent cloning of the cDNAs for the catalytic, protein kinase subunits of this complex, IKK-α and IKK-β (8–12). Several lines of evidence now indicate that IKK-α and IKK-β can be regulated by phosphorylation. The initial indications were that the IKK complex can be activated in vitro by the MAP3K MEKK1 (MAPK/ERK kinase kinase 1) (7) and that the complex, activated either in vitro by MEKK1 or in vivo by exposure of cells to TNF-α can be inactivated by phosphatase treatment (7, 8). Additional work then demonstrated that (i) mutation of potential phosphoacceptor residues to alanine in the activation loop of IKK-α or IKK-β abrogated activity (12), (ii) mutation of these same residues in IKK-β to the phosphoresidue mimic glutamic acid results in its constitutive activation (12), (iii) both IKK-α and IKK-β can be activated in vivo when overexpressed with MEKK1 or the related MAP3K NF-κB-inducing kinase (NIK) (10, 11, 13–15), and (iv) immunoprecipitated NIK can phosphorylate immunoprecipitated IKK-α (16). Therefore, an important conceptual advance in our understanding of NF-κB regulation is that it can be activated by protein kinase cascade, the core elements of this cascade being a MAP3K and an IKK (7, 10, 17).

These findings have already begun to provide a framework for understanding how NF-κB can be activated by diverse stimuli. For example, compelling evidence has been presented to show that NIK mediates the NF-κB-inducing activity of TNF-α (17, 18), whereas MEKK1 mediates the NF-κB-inducing activity of Tax (15). Thus, different stimuli can activate NF-κB by targeting different MAP3Ks.

These findings moreover raise the possibility that yet other MAP3Ks might activate NF-κB. MAP3Ks were originally identified as components of signaling cascades in which a MAP3K phosphorylates and activates a MAP2K, which in turn phosphorylates and activates a MAPK; the latter include the mitogen-activated ERK and the stress-activated c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase) and p38 families (19). Here we show that MEKK2 and MEKK3, but not certain other MAP3Ks, can activate NF-κB, and show that this activation occurs by their activation of IKK-α and IKK-β.

EXPERIMENTAL PROCEDURES

Plasmids—pCMV5-HA-MEKK2 (20), pCMV5-HA-MEKK3 (20), and pCMV5-ΔMEKK4 (21) were gifts of Dr. Gary Johnson (National Jewish Medical and Research Center). pcDNA3-ASK1 (22) was a gift of Dr. Hidori Ichijo (The Cancer Institute, Tokyo). pcDNA3-MLK3 was constructed by subcloning into the BamH I (blunt)/EcoRI site of pcDNA3 the 2.7-kilobase pair NcoI (blunt)/EcoRI coding sequence fragment of pPTK1–3.2 (23); the latter was a gift of Dr. Richard Spritz (University of Wisconsin-Madison). (PRDII)/E1bCAT, a reporter gene that contains three copies of the NF-κB binding site from the interferon-β enhancer,
an E1b promoter, and the CAT gene, was a gift of Dr. Tom Maniatis (Harvard University). The sources of p[PRDI]CAT, which contains two copies of the NF-κB binding site from the interferon-β enhancer, pCMV3-MEKK1, which encodes for a C-terminal 672-residue fragment of MEKK1 (24), and all other plasmids have been described (7, 14).

Tissue Culture and Transfection—HeLa cells were maintained as described (7). Transfections performed in 3.5-cm-diameter wells were conducted by calcium phosphate precipitation (25) or by using Fugene 6 according to the manufacturer's instructions (Boehringer Mannheim). CAT and protein measurements were performed as described (7, 26).

Immunoprecipitations—Cells were washed once with Dulbecco's phosphate-buffered saline containing 1 mM EDTA and then lysed by the addition of 1 ml of buffer B (14) containing 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. After centrifugation of the whole cell lysate at 16,000 × g for 10 min at 4 °C, the supernatant was incubated with 10 μl of M2-agarose with end over end rotation for 1 h at 4 °C. The resin was then washed three times with buffer B and eluted by the addition of 20 μl of 2× SDS-PAGE loading buffer.

Western Blotting—Immunoprecipitates were subjected to SDS-PAGE and then transferred to Immobilon-P membranes (Millipore). Membranes were blocked and then incubated with anti-IxBα (C-21, Santa Cruz Biotechnology), anti-phospho-Ser-32 IκBα, anti-phospho-Ser-32 IκBβ (New England Biolabs), anti-phospho-Ser-32 IκBα, anti-MAP3Ks other than MEKK1 or NIK might activate NF-κB, or empty expression vector. The Flag-tagged IκBα was then immunoprecipitated with anti-Flag antibodies and the kinase assay was carried out (21). The ATP concentration employed to initiate the IKK reactions was 50 μM instead of 200 μM. Kinase activities were quantitated using a Molecular Dynamics Storm 860 PhosphorImager.

RESULTS

MEKK2 and MEKK3 Induce NF-κB Activity and Site-specific Phosphorylation of IκBα—To examine the possibility that MAP3Ks other than MEKK1 or NIK might activate NF-κB HeLa cells were cotransfected with a reporter gene that contains two NF-κB binding sites and expression constructs for a series of MAP3Ks, including MEKK2 (20), MEKK3 (20), the catalytic domain of MEKK4 (ΔMEKK4) (21), apoptosis signal-regulating kinase 1 (ASK1) (22), and mixed-lineage kinase 3 (MLK3, also known as protein-tyrosine kinase 1 or SH3 domain-containing proline-rich kinase 23, 27, 28). All can activate the JNK pathway (20–22, 28). In addition, MEKK3, MEKK4, and ASK1 can activate the p38 pathway (22, 29, 30), whereas MEKK2 and MEKK3 can activate the ERK pathway (20). As shown in Fig. 1A, under conditions where overexpression of the positive controls MEKK1 and NIK induces activation of the NF-κB reporter gene, overexpression of MEKK3 (as reported previously; Ref. 31) and, to a lesser extent, MEKK2 do not induce activation as well. ΔMEKK4, ASK1, and MLK3 did not induce activation in either these cells (Fig. 1A) or the murine fibroblast cell line L929 (data not shown) but as expected did induce robust activation of coexpressed JNK1 in HeLa cells (data not shown).

NIK and MEKK1 activate NF-κB by inducing the site-specific phosphorylation of IκBα. In the case of IκBα, this phosphorylation, which occurs at Ser-32 and Ser-36, is manifested by slower mobility when IκBα is examined by SDS-PAGE (3, 4). To examine whether MEKK2 and MEKK3 might act through the same mechanism, HeLa cells were cotransfected with an expression constructs for Flag-tagged IκBα, MEKK2, or empty expression vector. As shown in Fig. 1A, MEKK2 and MEKK3 both induce the appearance of a more slowly migrating IκBα species (top panel, lanes 3 and 5, upper bands) that is abolished when an S32A/S36A IκBα mutant is examined (lanes 4 and 6), consistent with this species being N-terminally phosphorylated IκBα. ΔMEKK4, ASK1, and MLK3 did not induce the appearance of this more slowly migrating species (data not shown). This IκBα species was examined further by probing this blot with antibodies specific for phospho-Ser-32 IκBα. As shown in Fig. 1B (bottom panel, lanes 3 and 5), the slower migrating IκBα species induced by MEKK2 or MEKK3 is immunoreactive with these antibodies. We conclude that MEKK2 and MEKK3 can induce site-specific, N-terminal phosphorylation of IκBα in vivo.

MEKK2 and MEKK3 Activate Both IKK-α and IKK-β—Both MEKK1 and NIK induce site-specific phosphorylation of IκBα by activating IKK-α and IKK-β. To examine whether MEKK2 or MEKK3 acts by the same mechanism, HeLa cells were cotransfected with expression constructs for Flag-tagged IκBα, IKK-β, or empty expression vector. As shown in Fig. 1, both MEKK2 and MEKK3 both induce the appearance of a more slowly migrating IκBα species (top panel, lanes 3 and 5, upper bands) that is abolished when an S32A/S36A IκBα mutant is examined (lanes 4 and 6), consistent with

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**Fig. 1.** Activation of NF-κB and site-specific phosphorylation of IκBα induced by MEKK2 and MEKK3. A, HeLa cells were co-transfected by calcium phosphate precipitation with 3 μg of [PRDI]CAT and 6 μg of pCMV5-MEKK1, pCMV5-ΔMEKK4, pCMV5-MEKK3, pCMV5-MEKK2, pcDNA3-NIK, pcDNA3-ASK1, pcDNA3-MLK3, or pCMV5. Cells were harvested 41 h post-transfection. CAT activities were normalized to protein concentrations of extracts. Shown is a representative result, performed in duplicate with standard deviations, from three independent experiments. B, HeLa cells were cotransfected using Fugene 6 with 0.5 μg of expression vectors for wild-type (WT) (pCMV4-FlagIκBα) or mutant (M) (pCMV4-FlagIκBα (S22A/S36A)) IκBα, and 4 μg of pCMV5-HA-MEKK2, pCMV5-HA-MEKK3, or pCMV5. 24 h post-transfection, the epoxygenated IκBα was immunoprecipitated with M2-agarose and subjected to 12% SDS-PAGE. Top, immunoblotting (IB) was first performed with anti-IκBα antibodies (C-21, Santa Cruz Biotechnology). The positions of unphosphorylated (IκBα) and phosphorylated (P-IκBα) are indicated to the right, and those of molecular mass markers (in kDa) are shown on the left. Bottom, the immunoblot was then stripped and reprobed with anti-phospho-Ser-32 IκBα antibodies (New England Biolabs). Shown are representative results from three independent experiments.
MEKK2 and MEKK3 Activate IKK-α and IKK-β

The positions of IKK or JNK are indicated to the right. anti-JNK1 (C-17, Santa Cruz Biotechnology) (C). Whole cell extracts were prepared 24 h post-transfection and divided into two equal aliquots, and IKK or JNK from each aliquot was then immunoprecipitated with M2-agarose. One set of immunoprecipitates was then assayed for kinase activity (KA) toward GST-IκBα (5–55) (A, B, top) or GST-c-jun (1–79) (C, top) in the presence of [γ-32P]ATP and then analyzed by 12% SDS-PAGE and autoradiography. The positions of the substrates are indicated to the right. The relative degrees of substrate 32P incorporation are indicated below the gels. The other set of immunoprecipitates was subjected to SDS-PAGE and analyzed by immunoblotting (IB) using anti-Flag (D-8, Santa Cruz Biotechnology) (A and B, bottom) or anti-JNK1 (C-17, Santa Cruz Biotechnology) (C, bottom) antibodies. The positions of IKK or JNK are indicated to the right. Shown are representative results from three to four independent experiments.

FIG. 2. Activation of both IKK-α and IKK-β by MEKK2 and MEKK3. HeLa cells were cotransfected using Fugene 6 with 3 μg of pCMV5-MEKK1, pCMV5-HA-MEKK2, pCMV5-HA-MEKK3, pcDNA3-NIK, pcDNA3-MLK3, or pCMV5, and 0.5 μg of pRK-FlagIKK-α (A), pRK-FlagIKK-β (B), or pcDNA3-FlagJNK1 (C). Whole cell extracts were prepared 24 h post-transfection and divided into two equal aliquots, and IKK or JNK from each aliquot was then immunoprecipitated with M2-agarose. One set of immunoprecipitates was then assayed for kinase activity (KA) toward GST-IκBα (5–55) (A and B, top) or GST-c-jun (1–79) (C, top) in the presence of [γ-32P]ATP and then analyzed by 12% SDS-PAGE and autoradiography. The positions of the substrates are indicated to the right. The relative degrees of substrate 32P incorporation are indicated below the gels. The other set of immunoprecipitates was subjected to SDS-PAGE and analyzed by immunoblotting (IB) using anti-Flag (D-8, Santa Cruz Biotechnology) (A and B, bottom) or anti-JNK1 (C-17, Santa Cruz Biotechnology) (C, bottom) antibodies. The positions of IKK or JNK are indicated to the right. Shown are representative results from three to four independent experiments.

The potencies of MEKK1, MEKK2, MEKK3, and NIK in activating IKK-α, IKK-β, or a NF-κB reporter gene were analyzed in more detail (Fig. 3). All four MAP3Ks induce dose-dependent increases in the activities of coexpressed IKK-α or IKK-β. In the case of coexpressed IKK-α, the dose response curves are roughly comparable (Fig. 3A; see also Fig. 2A). In the case of coexpressed IKK-β, NIK is a somewhat less potent activator than the other MAP3Ks (Fig. 3B). In contrast, NIK is a substantially more potent activator of an NF-κB reporter gene than the other three MAP3Ks (Fig. 3C). For example, the NF-κB reporter gene activity induced by 40 ng of NIK expression vector is comparable or even greater than that induced by 4000 ng of expression vector for either MEKK1, MEKK2, or MEKK3.

Dominant Negative IKK-α and Dominant Negative IKK-β Inhibit MEKK2- and MEKK3-Induced NF-κB Activation—The experiments described above indicate that MEKK2 and MEKK3 can activate both IKK-α and IKK-β in vitro. To examine whether this activation is functionally significant, HeLa cells were cotransfected with expression constructs for MEKK1, MEKK2, MEKK3, or empty expression vector, expression constructs for dominant negative, catalytically inactive IKK-α (K44A), IKK-β (K44A), or empty expression vector, and an NF-κB reporter gene. As shown in Fig. 4, under conditions where activation of the NF-κB reporter gene induced by MEKK1 is almost completely inhibited by dominant negative IKK-α or dominant negative IKK-β (13, 14, 32), that induced by either MEKK2 and MEKK3 is completely abolished. This therefore provides evidence that IKK-α and IKK-β mediate the NF-κB inducing activity of MEKK2 and MEKK3.

FIG. 3. Dose response experiments examining IKK and NF-κB activation by MAP3Ks. HeLa cells were cotransfected using Fugene 6 with 1 μg of pRK-FlagIKK-α (A), 0.5 μg of pRK-FlagIKK-β (B), or 2 μg of (PRDH)E1bCAT (C); and 40, 400, or 4000 ng of pCMV5-MEKK1, pCMV5-HA-MEKK2, pCMV5-HA-MEKK3, or pcDNA3-NIK. The total DNA dose was brought up to 5 μg (A, 4.5 μg, or 6 μg (C) with pCMV5. A and B, IKK was immunoprecipitated with M2-agarose from whole cell extracts prepared 23-24 h post-transfection and assayed for activity toward GST-IκBα (5–55) in the presence of [γ-32P]ATP. IKK expression levels were analyzed by immunoblots of aliquots of the whole cell extracts using anti-Flag antibodies. C, cell extracts prepared 25 h post-transfection were assayed for CAT activity and normalized to the protein concentrations of the extracts. Shown are representative results from two to three independent experiments.

MEKK1, MEKK2, MEKK3, or empty expression vector, expression constructs for dominant negative, catalytically inactive IKK-α (K44A), IKK-β (K44A), or empty expression vector, and an NF-κB reporter gene. As shown in Fig. 4, under conditions where activation of the NF-κB reporter gene induced by MEKK1 is almost completely inhibited by dominant negative IKK-α or dominant negative IKK-β (13, 14, 32), that induced by either MEKK2 and MEKK3 is completely abolished. This therefore provides evidence that IKK-α and IKK-β mediate the NF-κB inducing activity of MEKK2 and MEKK3.

DISCUSSION

Here we identify two additional, previously cloned MAP3Ks: MEKK2 and MEKK3, which now join NIK and MEKK1 as activators of IKK and NF-κB, thereby enlarging our framework for understanding NF-κB activation. Such knowledge is essen-
MEKK2 and MEKK3 Activate IKK-α and IKK-β

Fig. 4. Inhibition of the NF-κB-inducing activity of MEKK2 and MEKK3 by dominant negative IKK-α and dominant negative IKK-β. HeLa cells were cotransfected using Fugene 6 with 1.5 μg of (PRDII)E1bCAT, 3 μg of pCMV5-MEK1, pCMV5-HA-MEK2, pCMV5-HA-MEK3, or pCMV5, and 1.5 μg of pRK-FlagIKK-α (K44A), pRK-FlagIKK-β (K44A), or pCMV5. Cells were harvested 25 h post-transfection. CAT activities were normalized to protein concentrations of extracts. Shown is a representative result, performed in duplicate with standard deviations, from two independent experiments.

The fact that many MAP3Ks have the capacity to activate distinct pathways now raises the problem of how specificity in signaling pathways is achieved. One example of this is provided by the observation that Tax activates MEKK1 and induces potent NF-κB activity (15) but only modest JNK activity (15, 36), despite the fact that MEKK1 overexpression coordinately activates both (7). Thus, the relative capacities of a MAP3K to activate distinct signaling pathways may be modulated in a manner that is stimulus-specific.

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