Cell, Vol. 88, 213–222, January 24, 1997, Copyright ©1997 by Cell Press

Activation of the IκBα Kinase Complex by MEKK1, a Kinase of the JNK Pathway

Frank S. Lee,* Jeremiah Hagler,* Zhijian J. Chen,† and Tom Maniatis*
*Department of Molecular and Cellular Biology
Harvard University
7 Divinity Avenue
Cambridge, Massachusetts 02138
†ProScript, Inc.
38 Sidney Street
Cambridge, Massachusetts 02139

Summary

Both NF-κB and c-Jun are activated by cytokines such as TNF-α and by stresses such as UV irradiation. A key step in the activation of NF-κB is the phosphorylation of its inhibitor, IκBα, by a ubiquitination-inducible multiprotein kinase complex (IκBα kinase). A central kinase in the c-Jun activation pathway is mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1). Here, we show that MEKK1 induces the site-specific phosphorylation of IκBα in vivo and, most strikingly, can directly activate the IκBα kinase complex in vitro. Thus, MEKK1 is a critical component of both the c-Jun and NF-κB stress response pathways. Since the IκBα kinase complex can be independently activated by ubiquitination or MEKK1-dependent phosphorylation, it may be an integrator of multiple signal transduction pathways leading to the activation of NF-κB.

Introduction

Exposure of cells to certain cytokines or environmental stresses leads to the activation of the transcription factors NF-κB and c-Jun. In unstimulated cells, NF-κB is sequestered in the cytoplasm in a complex with IκBα or another member of the IκB family of inhibitor proteins (reviewed by Baldwin, 1996). Following stimulation by a variety of inducers, NF-κB is activated as a consequence of the phosphorylation and subsequent proteolytic degradation of the IκB protein. IκBα is phosphorylated at Serine residues 32 and 36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995), an event that targets this protein for degradation by the ubiquitin-proteasome pathway (Chen et al., 1995; reviewed by Baldwin, 1996). Recent studies have identified a large, multisubunit complex in HeLa cell cytoplasmic extracts that can phosphorylate IκBα at Ser-32 and -36 (the IκBα kinase, Chen et al., 1996). A novel property of this kinase, which was isolated from unstimulated cells, is that it can be activated in vitro by ubiquitination.

A second branch of the stress response is the c-Jun N-terminal kinase (JNK) pathway (also known as the stress-activated protein kinase pathway; for recent reviews and references see Karin, 1995, and Kyriakis and Avruch, 1996). The JNK cascade can be activated by small GTP-binding proteins that include Cdc42 and Rac1 (Coso et al., 1995; Minden et al., 1995), and protein kinases that they directly activate, such as PAK (Bagrodia et al., 1995). MEKK1 (Lange-Carter et al., 1993) is then activated by these proteins by a mechanism yet to be determined. MEKK1 then activates MKK4, which in turn activates JNK (Sanchez et al., 1994; Yan et al., 1994; Derijard et al., 1995). Among the substrates of JNK are c-Jun, ATF-2, and Elk-1 (Derijard et al., 1994; Kyriakis et al., 1994; Gupta et al., 1995; Whitmarsh et al., 1995).

While the signal transduction cascade leading to the activation of JNK is relatively well defined, the steps leading to the phosphorylation of IκBα are poorly understood. Many of the stimuli that induce NF-κB, such as TNF-α, UV irradiation, and lipopolysaccharide, also activate the JNK cascade, thereby raising the possibility that the two pathways utilize common signal transduction components. Indeed, transfection of MEKK1 induces the degradation of IκBα and activates an NF-κB reporter gene (Hirano et al., 1996; Meyer et al., 1996), suggesting a link between the NF-κB and JNK pathways. These observations raise two critical questions. First, is MEKK1-induced degradation mediated by the same site-specific phosphorylation of IκBα identified in other contexts? Second, and more importantly, what is the target of MEKK1 in the NF-κB pathway? Here, we show that MEKK1 does in fact induce phosphorylation of IκBα at its sites of regulatory phosphorylation and that this occurs by direct activation of the IκBα kinase. Thus, MEKK1 is a critical coordinate regulator of both the NF-κB and JNK pathways.

Results

IκBα Kinase Activity Is Inducible by TNF-α

Previous studies left open the question of whether the IκBα kinase is regulated by inducers of NF-κB. In those studies, the IκBα kinase was detected as an apparently constitutive activity in S100 cytoplasmic extracts prepared from uninduced HeLa cells using the hypotonic lysis procedure of Dignam et al., 1983 (Chen et al., 1996). Using an alternative method for preparing cytoplasmic extracts (a rapid lysis procedure detailed in Experimental Procedures), we now find that the IκBα kinase activity is inducible by TNF-α. HeLa cells were treated with TNF-α for differing lengths of time, and the rapid lysis extracts assayed for the presence of endogenous IκBα by Western blotting and for IκBα kinase activity by incubation with exogenous ^{35}S-labeled IκBα in the presence of okadaic acid (Figures 1A and 1B). We note that in these and all subsequent experiments okadaic acid is employed strictly as a phosphatase inhibitor (i.e., to preserve the phosphorylated IκBα species) rather than as an inducer of IκBα phosphorylation (Thevenin et al., 1990; Traenckner et al., 1995). Consistent with previous results (Henkel et al., 1993; Mellits et al., 1993), extracts from uninduced HeLa cells contain hypophosphorylated IκBα (Figure 1A, lane 1), but after only 5 min of TNF-α treatment a significant portion of the endogenous IκBα is phosphorylated (as revealed by the slower migrating IκBα species, lane 2). After 30 min of treatment, virtually

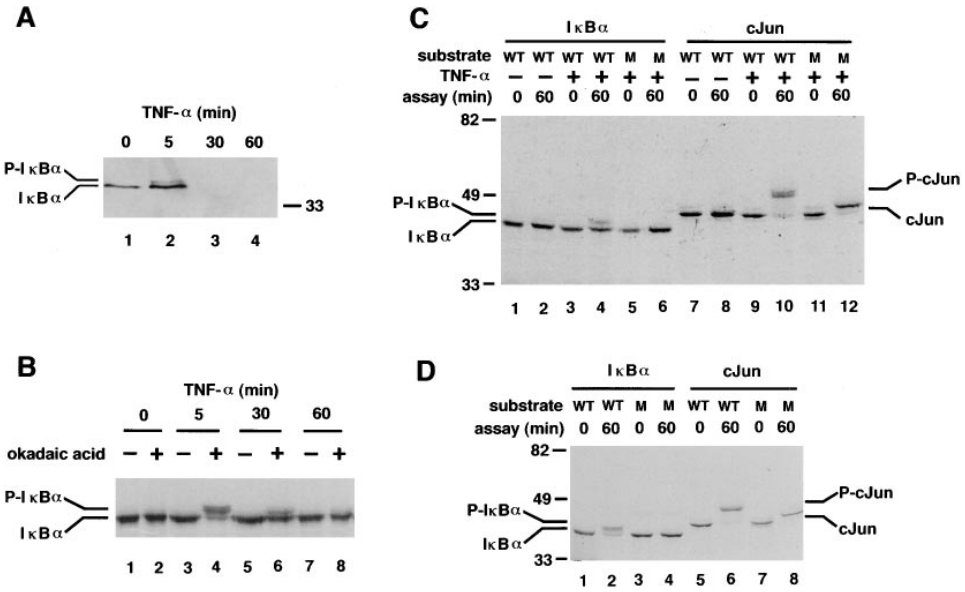

Figure 1. IκBα Kinase Activity Is Inducible and Is Correlated With JNK Activity

(A) HeLa cells were treated with TNF-α for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (14 μg) were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-IκBα antibodies. The positions of unphosphorylated (IκBα) and phosphorylated (P-IκBα) IκBα are indicated to the left. Molecular weight marker (in kilodaltons) is indicated to the right.

(B) HeLa cells were treated with TNF-α for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (9 μg) were then incubated with ^{35}S-labeled IκBα in the absence or presence of 6 μM okadaic acid for 1 hr at 30°C. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The positions of unphosphorylated (IκBα) and phosphorylated (P-IκBα) IκBα are indicated to the left.

(C) HeLa cells were either mock or TNF-α (5 min) treated, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (8 μg) were then incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left. The positions of unphosphorylated (cJun) and phosphorylated (P-cJun) c-Jun are indicated to the right; those for IκBα are indicated to the left.

(D) HeLa cell S100 extracts (18 μg) were incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

all of the IκBα is degraded (lane 3). Parallel assays with the same extracts reveal that the IκBα kinase activity is absent in uninduced cells (Figure 1B, lane 2) (activity is weakly detectable when higher concentrations of these extracts are employed). However, after exposure of cells to TNF-α for only 5 min, IκBα kinase activity can be readily detected, as evidenced by the slower-migrating IκBα species (lane 4). Fractionation of these extracts by gel filtration reveals that the TNF-α-inducible IκBα kinase activity resides in a large (approximately 700 kDa) complex (data not shown). Interestingly, this activity persists and is present after 30 min of TNF-α induction (lane 6), a time at which the endogenous IκBα has been degraded (Figure 1A, lane 3). IκBα kinase activity is essentially absent at 60 min (Figure 1B, lane 8). It is formally possible that the IκBα kinase activity is constitutive and that TNF-α treatment simply results in the inactivation of a phosphatase in the extract that dephosphorylates IκBα. To address this possibility, the same extracts were incubated with ^{35}S-labeled IκBα in the absence of okadaic acid (Figure 1B, lanes 1, 3, 5, and 7). Under these conditions, the IκBα mobility shift is completely abolished (for example, compare lanes 3 and 4). Thus, the effects of TNF-α treatment cannot be accounted for solely by inactivation of an okadaic acid–sensitive IκBα phosphatase, implying that TNF-α treatment induces IκBα kinase activity. Furthermore, the rapid induction of IκBα kinase activity correlates with the rapid appearance of the phosphorylated form of IκBα.

Coordinate Activation of IκBα Kinase and JNK Activities In Vitro

TNF-α treatment also leads to the activation of c-Jun by JNK (Hibi et al., 1993). We therefore carried out experiments to determine whether the IκBα kinase and JNK are coactivated in extracts from TNF-α-treated cells. Cytoplasmic extracts from uninduced and TNF-α-induced HeLa cells were incubated with in vitro translated, ^{35}S-labeled IκBα or c-Jun, and the proteins fractionated by SDS-PAGE (Figure 1C). As before, IκBα kinase activity is detected in extracts from TNF-α-induced cells but not in those from uninduced cells (compare lanes 2 and 4). The specificity of phosphorylation is indicated by the fact that the S32A/S36A mutation in IκBα completely abolishes the IκBα shift (lane 6). Similarly, extracts from TNF-α-induced cells show JNK activity, as evidenced by the appearance of a c-Jun species with markedly reduced mobility (compare lanes

9 and 10). The observed shift is a result of JNK activity, since amino acid substitutions at the sites of JNK phosphorylation (S63A/S73A) in c-Jun abolish this shift (lane 12). We note that a distinct shift is observed with the c-Jun mutant, suggesting that JNK may phosphorylate c-Jun at residues other than Ser-63 and -73. Importantly, extracts from uninduced cells show no significant JNK activity (lane 8). Thus, both the IκBα kinase and JNK activities are activated in the rapid lysis extracts prepared from TNF-α-treated, but not untreated, cells.

By contrast, the IκBα kinase activity is readily detected in S100 cytoplasmic extracts prepared from unstimulated HeLa cells using the hypotonic lysis procedure (Chen et al., 1995). It is possible that stress pathways are activated by this procedure, since another form of osmotic stress, hyperosmolar shock, has been shown to be an efficient activator of the JNK pathway (Galcheva-Gargova et al., 1994). Indeed, both the IκBα kinase and the JNK activities were detected when the S100 extracts were incubated for 60 min (Figure 1D). A time-dependent activation of JNK was detected when the S100 extracts were incubated and then examined by an in-gel kinase assay employing the JNK substrate ATF-2 (data not shown). Thus, both the JNK and IκBα kinase may be activated during incubation of the S100 extracts, possibly owing to the hypotonic lysis conditions.

MEKK1 Activates NF-κB In Vivo

Transient transfection studies were conducted to examine the relationship between the activation of the IκBα kinase and JNK in vivo. The IFN-β enhancer contains multiple positive regulatory domains (PRDs) that bind distinct transcription factors, including NF-κB (PRDII) and ATF-2/c-Jun (PRDIV) (reviewed in Thanos et al., 1993). HeLa cells were transfected with reporters linked to either two copies of PRDII (PII), six copies of PRDIV (PIV), or the intact IFN-β enhancer (IFN), which includes these as well as other PRDs, and either an expression vector for MEKK1 or an expression vector alone. Note that in these and all subsequent experiments, MEKK1 and MEKK1Δ refer to the 672 and 321 residue C-terminal fragments, respectively, of the full-length molecule (for discussion, see Xu et al., 1996). Both kinases are constitutively active and indistinguishable in transfection studies. As expected, MEKK1 activates the reporter linked to a multimer of PRDIV (Figure 2A), which binds to either an ATF-2 homodimer or an ATF-2/c-Jun heterodimer (Du et al., 1993). Both ATF-2 and c-Jun contain transcriptional activation domains that are phosphorylated by the JNK pathway (Gupta et al., 1995). Importantly, MEKK1 also activates the PRDII reporter. MEKK1 does not activate all promoters, since its effect on a reporter gene containing the intact IFN-β enhancer is only marginal. This enhancer contains additional PRDs that bind factors other than NF-κB or ATF-2/c-Jun (see Thanos et al., 1993). As expected, the IFN-β enhancer is effectively activated by virus infection, which activates all of the PRDs. We conclude that MEKK1 can activate both ATF-2/c-Jun and NF-κB in vivo.

To examine whether MEKK1 plays a role in the activation of NF-κB in response to TNF-α, HeLa cells were transfected with a PRDII reporter and expression vector for catalytically inactive (K432M) MEKK1Δ, or empty expression vector. Some cells were then stimulated with TNF-α, and subsequently all cells were harvested and examined for reporter gene activity. As expected, TNF-α activates the PRDII reporter efficiently (Figure 2B). By contrast, the mutant MEKK1Δ (K432M) inhibits both the basal and TNF-α-induced activity of this reporter, thus behaving as a dominant negative inhibitor, as has also been shown by Hirano et al. (1996). Similar results are observed in L929 cells (Figure 2C). As a negative control, cAMP activation of a cAMP response element reporter is not significantly affected by dominant negative MEKK1Δ (Figure 2D). These results suggest that MEKK1 plays a role in TNF-α activation of NF-κB.

MEKK1 Activation of NF-κB Occurs through Site-Specific Phosphorylation of IκBα

Numerous stimuli that activate NF-κB have been shown to induce site-specific phosphorylation of IκBα at Ser-32 and -36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). Experiments were therefore conducted to examine whether MEKK1 induces this same phosphorylation. HeLa cells were transfected with

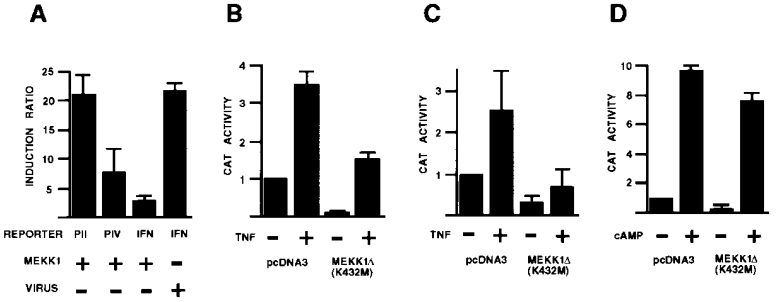

Figure 2. MEKK1 Activates NF-κB In Vivo

(A) HeLa cells were transfected with 3 μg of $(PRDII)_2$CAT, $(PRDIV)_6$CAT or -110IFN-βCAT, 2 μg pCMV-lacZ, and 4 μg of pCMV5-MEKK1 or pcDNA3. Twenty-six to 28 hr posttransfection, cells in one well were infected with Sendai virus for 15 hr. All cells were harvested 41 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

(B and C) HeLa (B) or L929 (C) cells were transfected with 3 μg of $(PRDII)_2$CAT, 2 μg pCMV-lacZ, and 4 μg of pcDNA3-FlagMEKK1Δ (K432M) or pcDNA3. Forty to 41 hr posttransfection, some cells were treated with 20 ng/ml mouse TNF-α (Boehringer) for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for β-galactosidase. Shown are the averages and standard deviations from (B) one experiment performed in triplicate or (C) three independent experiments.

(D) L929 cells were transfected with 3 μg of $(CRE)_6$CAT, 2 μg pCMV-lacZ, and 4 μg of pcDNA3-FlagMEKK1Δ (K432M) or pcDNA3. Forty to 41 hr posttransfection, some cells were treated with 1 mM 8-Br-cAMP for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for β-galactosidase. Shown are the averages and standard deviations from three independent experiments.

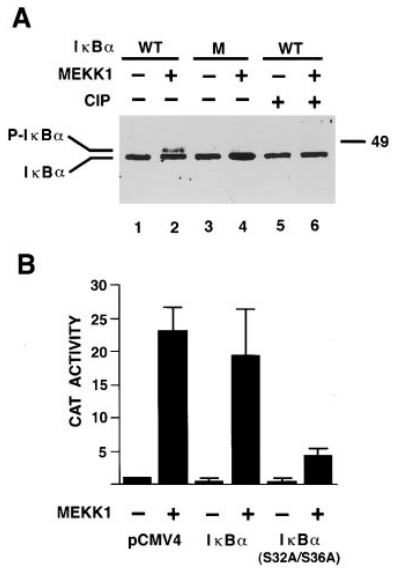

Figure 3. MEKK1 Activation of NF-κB Is through Site-Specific Phosphorylation of IκBα

(A) HeLa cells were transfected with 0.3 μg of expression vectors for wild-type (WT) (pCMV4-FlagIκBα) or mutant (M) (pCMV4-FlagIκBα [S32A/S36A]) IκBα, 3 μg of pCMV5-MEKK1 or pCMV5, and 3 μg of SP72. Forty-one hr posttransfection, epitope-tagged IκBα was immunoprecipitated, and some samples were treated with calf intestinal alkaline phosphatase (CIP). All samples were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-IκBα antibodies.

(B) HeLa cells were transfected with 3 μg of $(PRDII)_2$CAT, 10 ng of pCMV4-FlagIκBα, pCMV4-FlagIκBα (S32A/S36A) or pCMV4, and 4 μg of pCMV5-MEKK1 or pCMV5. Cells were harvested 38 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

expression vectors for Flag-tagged wild-type or mutant (S32A/S36A) IκBα, and an expression vector for MEKK1 or the expression vector alone. IκBα was then immunoprecipitated with anti-Flag antibodies, and then visualized by Western blotting using anti-IκBα antibodies. MEKK1 induces the appearance of an IκBα species with reduced mobility compared to that isolated from uninduced cells (Figure 3A, lane 2). This species is sensitive to treatment with calf intestinal alkaline phosphatase (compare lanes 2 and 6), consistent with its being a phosphorylated form of IκBα. Most importantly, Ser-to-Ala mutations at residues 32 and 36 of IκBα abolish this species (lane 4). Thus, MEKK1 induces site-specific phosphorylation of IκBα at Ser-32 and -36.

If the MEKK1-dependent phosphorylation of these serine residues is functionally important, their substitution by alanines should make IκBα degradation and hence NF-κB activation refractory to MEKK1 stimulation. To test this possibility, HeLa cells were transfected with the PRDII reporter gene and expression vectors for wild-type or mutant (S32A/S36A) IκBα or expression vector alone and either expression vector for MEKK1 or expression vector alone. HeLa cells transfected with wild-type IκBα display MEKK1-inducible reporter gene activity that is virtually the same as that of control (Figure 3B). In contrast, HeLa cells transfected with mutant IκBα display MEKK1-inducible activity that is significantly diminished. Other experiments indicate that it is necessary to transfect approximately 10-fold higher amounts of wild-type IκBα plasmid in order to observe a comparably reduced MEKK1-inducible reporter gene activity (data not shown). These experiments therefore indicate that site-specific phosphorylation of IκBα at Ser-32 and -36 plays a critical role in the MEKK1-dependent activation of NF-κB.

MEKK1 Coordinately Activates the IκBα Kinase and JNK In Vitro

The transfection data show that MEKK1 expression leads to the site-specific phosphorylation of IκBα. To investigate the possibility that MEKK1 activates the IκBα kinase, cytoplasmic extracts were prepared from uninduced HeLa cells by the rapid lysis procedure and then treated with recombinant MEKK1Δ. In the absence of MEKK1Δ, these extracts show no significant site-specific IκBα kinase or JNK activity when incubated with in vitro-translated, ^{35}S-labeled IκBα or c-Jun, respectively (Figure 4A, lanes 2 and 6). By contrast, when recombinant MEKK1Δ was added to the extract, site-specific phosphorylation of c-Jun was observed (compare lanes 7 and 9). Importantly, site-specific IκBα kinase activity was also observed (compare lanes 3 and 5), but MEKK1Δ alone fails to induce this site-specific phosphorylation (lane 1). To rule out the possibility that MEKK1Δ inactivates an IκBα phosphatase, extracts were incubated with MEKK1Δ in the absence or presence of the phosphatase inhibitor okadaic acid (Figure 4B). In the absence of okadaic acid, the MEKK1Δ-induced IκBα shift is largely abolished (compare lanes 2 and 3). Thus, the effects of MEKK1Δ cannot be accounted for solely by the inactivation of an okadaic acid–sensitive IκBα phosphatase, implying that MEKK1Δ activates the IκBα kinase. We conclude that MEKK1Δ coordinately activates the IκBα kinase and JNK pathways in cytoplasmic extracts.

MEKK1 Directly Activates the IκBα Kinase

In the JNK pathway, MEKK1 phosphorylates and activates MKK4, which, in turn, activates JNK. It is therefore possible that IκBα could be a substrate for MEKK1, MKK4, or JNK. When expressed as recombinant proteins, however, neither MKK4 nor JNK1 phosphorylated IκBα, with appropriate control experiments demonstrating that these proteins were enzymatically active (data not shown). MEKK1Δ did phosphorylate IκBα directly; however, the degree of phosphorylation was over 10-fold less than that seen with MKK4 as a substrate, and, as shown below, MEKK1Δ does not phosphorylate IκBα at Ser-32 or -36. In addition, recent experiments indicate the IκBα kinase activity resides in a large, approximately 700 kDa, multiprotein complex (Chen et al., 1996), and Western blotting of this complex fails to reveal the presence of MEKK1, MKK4, JNK1, or JNK2 (data not shown). A reasonable hypothesis, therefore, is that MEKK1 or one of the downstream kinases phosphorylates IκBα indirectly by stimulating the IκBα kinase.

To distinguish between these possibilities, MEKK1Δ was incubated with purified, ubiquitination-inducible

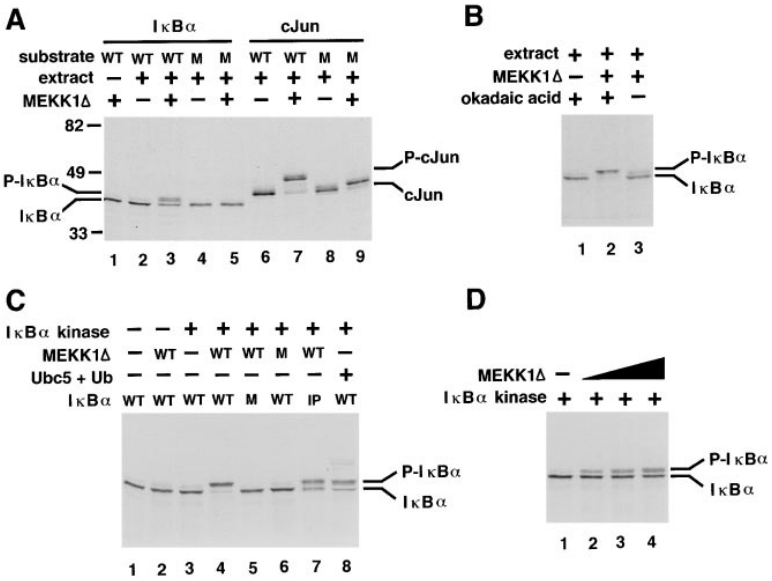

Figure 4. MEKK1 Directly Activates the IκBα Kinase

(A) Uninduced HeLa cell cytoplasmic extracts (2 μg) prepared by the rapid lysis procedure were incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun in the absence or presence of 20 ng MEKK1Δ for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1Δ and ^{35}S-labeled IκBα in the absence of extract. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

(B) Uninduced HeLa cell cytoplasmic extracts (2 μg) prepared by the rapid lysis procedure were incubated with ^{35}S-labeled IκBα in the absence or presence of 20 ng MEKK1Δ and/or 2.5 μM okadaic acid for 1 hr at 30°C. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(C) Purified IκBα kinase was incubated with wild-type (WT), mutant (S32A/S36A) (M), or immunoprecipitated wild-type (IP) ^{35}S-labeled FlagIκBα in the absence or presence of 20 ng wild-type (WT) or mutant (K432M) (M) MEKK1Δ, or 0.9 μg GST-Ubc5 + 0.5 mg/ml ubiquitin for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1Δ and ^{35}S-labeled FlagIκBα in the absence of IκBα kinase. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. In lane 8, additional bands at higher molecular weights than phosphorylated IκBα represent ubiquitinated IκBα species, owing to the presence of ubiquitination components (Chen et al., 1995, 1996).

(D) Purified IκBα kinase in the absence or presence of 5, 10, or 20 ng MEKK1Δ was incubated with ^{35}S-labeled FlagIκBα for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

IκBα kinase (Chen et al., 1996) and in vitro–translated, ^{35}S-labeled IκBα (Figure 4C). In the absence of the ubiquitin-conjugating enzyme Ubc5 and ubiquitin, the IκBα kinase is inactive (lane 3), while in their presence the kinase is active, as evidenced by the shift in mobility of the ^{35}S-labeled IκBα (lane 8), as shown previously (Chen et al., 1996). Strikingly, addition of MEKK1Δ independently activates the IκBα kinase (lane 4), while MEKK1Δ alone does not site-specifically phosphorylate IκBα (lane 2). That this shift reflects phosphorylation of IκBα at Ser-32 and -36 is indicated by the fact that the S32A/S36A mutant fails to display this shift (lane 5). The activation of the IκBα kinase depends on the catalytic activity of MEKK1Δ, since mutant MEKK1Δ(K432M) fails to activate (lane 6). Neither recombinant MKK4 nor JNK1 augments MEKK1Δ stimulation of the IκBα kinase (data not shown), ruling out the possibility that MEKK1Δ activation of the IκBα kinase is mediated by an insect (Sf9) cell MKK4- or JNK-like activity copurifying in trace amounts with the MEKK1Δ protein. To eliminate the possibility that MEKK1Δ activation of the IκBα kinase is mediated through a factor present in the wheat germ extract employed for in vitro translation of IκBα, immunoprecipitated IκBα was employed as a substrate. As shown in lane 7, this IκBα is also a substrate for MEKK1Δ-activated IκBα kinase. We conclude that MEKK1Δ activation of the IκBα kinase is direct. Additional experiments indicate that MEKK1Δ is a potent activator of the IκBα kinase (Figure 4D), with activation demonstrable with MEKK1Δ doses as low as 5 ng (lane 2). Finally, IκBα complexed with RelA (p65) is a substrate for MEKK1Δ-activated IκBα kinase, just as it is for the ubiquitination-activated kinase (data not shown).

The MEKK1-Inducible IκBα Kinase Is a High Molecular Weight Complex

To further examine the relationship between the MEKK1Δ- and the ubiquitination-inducible IκBα kinase previously reported (Chen et al., 1996), we fractionated HeLa cell cytoplasmic extracts and assayed for both activities (Figure 5). Notably, both MEKK1Δ- and ubiquitination-inducible IκBα kinase activities copurify during the first four steps of fractionation, which include ion exchange chromatography, ammonium sulfate fractionation, hydroxylapatite chromatography (data not shown), and gel filtration (Figures 5A and 5B). With regard to the gel filtration step, the peak of MEKK1Δ-inducible IκBα kinase activity elutes at a position (fractions 19 to 20) corresponding to a native molecular weight of approximately 700 kDa, indistinguishable from that of the ubiquitination-inducible IκBα kinase (Chen et al., 1996). Further fractionation by anion exchange chromatography reveals that the MEKK1Δ-inducible IκBα kinase activity elutes in a broader peak than the ubiquitination-inducible activity (Figures 5C and 5D). Thus, some fractions (e.g., 32 and 33) are inducible by both MEKK1Δ and ubiquitination, while others (e.g., 29 and 30) are inducible only by MEKK1Δ. We conclude that the two kinase complexes are largely similar but may have subtle differences in structure or subunit composition.

MEKK1 Is a Selective Activator of the IκBα Kinase

To examine the specificity of MEKK1Δ activation of the IκBα kinase, three additional kinases, casein kinase II (CKII), protein kinase A (PKA), and protein kinase Cζ (PKCζ), were assayed for their capacity to activate the

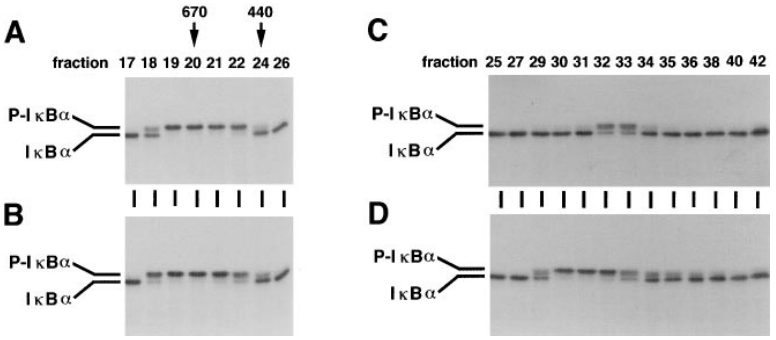

Figure 5. The MEKK1-Inducible IκBα Kinase Is a High Molecular Weight Complex

HeLa cell cytoplasmic extracts were fractionated as described in Experimental Procedures and then chromatographed on (A and B) a Superdex-200 gel filtration column followed by a (C and D) Mono-Q ion exchange column. Fractions were assayed for IκBα kinase activity with ^{35}S-labeled FlagIκBα in the presence of either (A and C) ubiquitination components or (B and D) 10 ng MEKK1Δ for 1 hr at 37°C in the presence of 3 μM okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The numbers 670 and 440 in (A) and (B) indicate elution positions of molecular weight standards (in kilodaltons).

IκBα kinase (Figure 6). In marked contrast to MEKK1Δ, none of these enzymes activates the IκBα kinase (Figure 6A). The enzymatic activity of the kinases is demonstrated by their roughly comparable degree of phosphorylation of recombinant IκBα with [γ-^{32}P]ATP (Figure 6B). The experiment shown in Figure 6A also demonstrates that none of the enzymes, aside from the IκBα kinase, phosphorylates IκBα at Ser-32 or -36 under the conditions employed. Phosphorylation by these other enzymes presumably occurs at residues other than Ser-32 or -36.

MEKK1 Activates the IκBα Kinase Complex by Phosphorylation

The fact that the catalytically inactive MEKK1Δ does not activate the IκBα kinase (Figure 4C, lane 6) strongly suggests that MEKK1Δ phosphorylates the IκBα kinase complex. To further examine this possibility, MEKK1Δ-activated IκBα kinase was incubated with or without calf intestinal alkaline phosphatase, and the IκBα kinase was then assayed for activity against ^{35}S-labeled IκBα in the absence or presence of MEKK1Δ. As shown in Figure 7A, treatment of the MEKK1Δ-activated IκBα kinase with phosphatase results in inactivation of IκBα kinase activity (compare lanes 2 and 4). Subsequent addition of MEKK1Δ results in substantial, though incomplete, restoration of IκBα kinase activity (compare lanes 2, 3, and 4). To extend these observations, the purified IκBα kinase was incubated with or without MEKK1Δ in the presence of [γ-^{32}P]ATP (Figure 7B). In the absence of MEKK1Δ, ^{32}P label was incorporated into three subunits (approximately 200, 180, and 120 kDa) of the IκBα kinase complex (lane 2). In the presence of MEKK1Δ, ^{32}P label was incorporated into three additional subunits of molecular weights of approximately 105, 64, and 54 kDa (lane 3). In conjunction with the experiment employing the catalytically inactive MEKK1Δ (Figure 4C), these experiments show that MEKK1Δ activates the IκBα kinase complex by phosphorylation. We are currently investigating which subunit(s) is the substrate(s) for MEKK1.

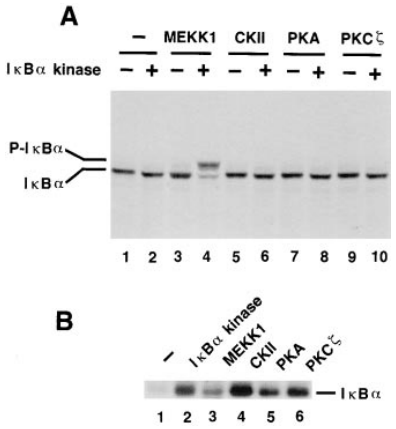

Figure 6. MEKK1 Is a Selective Activator of the IκBα Kinase

(A) MEKK1Δ (10 ng), CKII (0.35 ng, 250 mU, New England Biolabs), PKA (0.8 ng, 1 mU, New England Biolabs), and PKCζ (15 ng, PanVera), either alone or in combination with purified IκBα kinase, were incubated with ^{35}S-labeled FlagIκBα for 30 min at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 2) contained purified IκBα kinase and ^{35}S-labeled FlagIκBα. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(B) Purified IκBα kinase, MEKK1Δ, CKII, PKA, and PKCζ in the amounts employed in (A) were incubated with 0.5 μg $(His)_6$IκBα in the presence of [γ-^{32}P]ATP. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Relative kinase activities determined by phosphorimager analysis for the IκBα kinase, MEKK1Δ, CKII, PKA, and PKCζ are 1, 0.6, 2.2, 1.0, and 1.3, respectively.

Discussion

The activation of the IκBα kinase and JNK pathway by a single protein, MEKK1, provides a compelling explanation for how multiple stimuli can simultaneously activate these two distinct kinases (Figure 8). Thus, TNF-α, UV irradiation, and lipopolysaccharide have all been shown to activate the JNK pathway. Their implied activation of MEKK1 now provides a mechanism for the activation of the IκBα kinase. Stimuli such as phorbol myristate acetate/ionomycin could also potentially act through this pathway; in T cells, for example, phorbol myristate acetate and ionomycin synergistically activate the JNK pathway (Su et al., 1994) and thus may activate the IκBα kinase through MEKK1. The coordinate activation of the IκBα kinase and JNK raises the possibility that potential upstream activators of MEKK1, such as the small GTP-binding proteins Rac1, Cdc42, and Ras, as well as protein kinases that they activate, such as PAK, may also be common elements of a single upstream signal transduction mechanism.

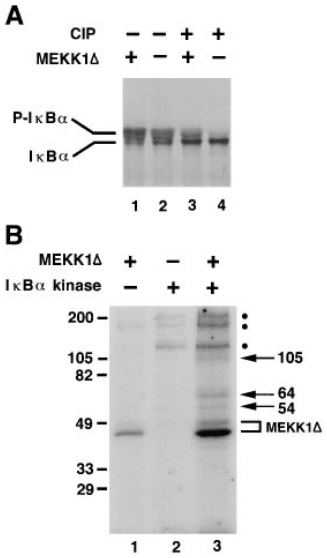

Figure 7. MEKK1 Activates the IκBα Kinase Complex by Phosphorylation

(A) MEKK1Δ-activated IκBα kinase was incubated with or without calf intestinal alkaline phosphatase (CIP, as indicated), and subsequently incubated with or without 12 ng MEKK1Δ (as indicated) and with ^{35}S-labeled FlagIκBα for 60 min at 37°C in the presence of 3 μM okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The doublet above the IκBα probably represents phosphorylation at one or both serines at positions 32 and 36.

(B) MEKK1Δ and purified IκBα kinase, either alone or in combination, were incubated in the presence of [γ-^{32}P]ATP. Reaction products were subjected to 8% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kDa) are shown to the left. Dots indicate bands (approximately 200, 180, and 120 kDa) present when the IκBα kinase is incubated with [γ-^{32}P]ATP in the absence of MEKK1Δ. Bracket indicates bands present when MEKK1Δ is incubated with [γ-^{32}P]ATP in the absence of the IκBα kinase, showing MEKK1Δ autophosphorylation.

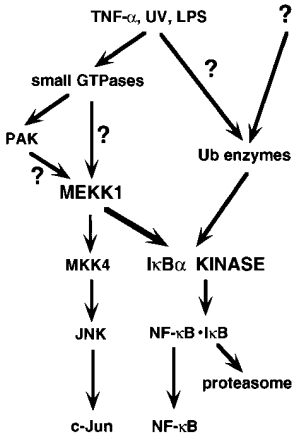

Figure 8. Model Showing Coordinate Activation of the IκBα Kinase and the JNK Pathway by MEKK1

Stressful stimuli such as TNF-α, UV irradiation, and lipopolysaccharide (LPS) activate MEKK1 by as-yet-to-be-identified mechanisms that involve small GTPases such as Rac1, Cdc42, and Ras and possibly the protein kinase PAK. MEKK1, in turn, coordinately activates the JNK pathway—which ultimately phosphorylates c-Jun—and the IκBα kinase. The IκBα kinase phosphorylates IκBα while still bound to NF-κB, targeting IκBα for degradation by the ubiquitin-proteasome pathway and thereby liberating NF-κB. The IκBα kinase can independently be activated by ubiquitination (Ub) enzymes (see Chen et al., 1996).

Previous studies have implicated kinases other than MEKK1 in the activation of NF-κB. For example, PKA has been shown to dissociate the NF-κB–IκB complex (Ghosh and Baltimore, 1990), while PKCζ coimmunoprecipitates with a factor that can phosphorylate IκBα (Diaz-Meco et al., 1994); with regard to the latter, it has been suggested that PKCζ activates a kinase that phosphorylates IκBα. Neither PKA nor PKCζ, however, phosphorylates IκBα at Ser-32 and-36, nor does either activate the IκBα kinase (Figure 6A). Additional kinases that have been implicated in NF-κB activation are raf-1 and the double-stranded RNA-activated protein kinase (PKR) (Finco and Baldwin, 1993; Yang et al., 1995). In preliminary experiments, we have been unable to observe activation of the IκBα kinase by enzymatically active c-raf (UBI) (data not shown). Additional studies will be required to determine the roles of raf and PKR in NF-κB regulation.

MEKK1 is a member of a family of enzymes that share a conserved C-terminal catalytic domain and may thus share overlap in substrates (Lange-Carter et al., 1993; Blank et al., 1996; Xu et al., 1996). Hence, it is conceivable that MEKK isoforms other than MEKK1 can activate the IκBα kinase. Different MEKK isoforms could potentially be involved in signaling responses to different stimuli. For example, MEKK1 has been shown to bind Ras in a GTP-dependent manner and thus its activity may be regulated in a similar fashion (Russell et al., 1995). Indeed, Ha-Ras activation of NF-κB (Devary et al., 1993) could be mediated, in part, by activation of MEKK1. Aside from its interaction with Ras, little is known about the regulation and activation of MEKK1. The recent identification of MEKK1 as a large membrane-associated protein, with its C-terminal catalytic domain constituting less than 20% of the molecule, raises the possibility of complex modes of regulation (Xu et al., 1996). While yet to be demonstrated, other MEKKs could conceivably be regulated by other upstream regulators such as Rac1, Cdc42, and PAK. These data also leave open the possibility that there may be other IκBα kinases that respond to stimuli distinct from those that signal through MEKK1.

We as well as others (Hirano et al., 1996) have shown that dominant negative MEKK1 inhibits TNF-α activation of an NF-κB reporter gene in vivo. This is in contrast to a recent report that reveals no effect of dominant negative MEKK1 in similar experiments, which conclude that MEKK1 lies on a pathway distinct from that of the IκBα kinase (Liu et al., 1996). At present, we have no explanation for this discrepancy. However, our transfection results are strongly supported by the observation that the IκBα kinase is phosphorylated and activated by MEKK1Δ in vitro. We thus conclude that the IκBα kinase and MEKK1, or minimally an MEKK isoform, are indeed part of the same pathway.

The IκBα kinase can be activated by ubiquitination independently of phosphorylation (Figure 8). To our knowledge, this dual regulation by phosphorylation or ubiquitination is unprecedented. Thus, the IκBα kinase itself can be considered a signal integrator, responding to both phosphorylation and ubiquitination. Different stimuli may therefore activate one, the other, or both

pathways. In principle, then, it may be possible to isolate an induced IκBα kinase species that is not ubiquitination-dependent, or one that is not phosphorylation-dependent. Indeed, the fractionation of highly purified IκBα kinase by ion exchange chromatography reveals kinase species that are phosphorylation but not ubiquitination inducible (Figures 5C and 5D).

A puzzling result from previous studies is that while IκBα kinase is easily assayed when present in HeLa cell S100 cytoplasmic extracts, the purified kinase is inactive, requiring ubiquitination components for activity (Chen et al., 1996). One possibility is that purification of the kinase separates the ubiquitination components from the kinase; hence, the purified kinase is inactive. The results described here raise a second and distinct possibility, namely that the IκBα kinase is activated by MEKK1 in the extract during the course of assay for IκBα kinase activity. Thus, purification of the IκBα kinase from S100 extracts removes it from both the ubiquitination components and MEKK1 present in the extract; in fact, Western blotting indicates that MEKK1 is not present in the IκBα kinase complex (data not shown). Either ubiquitination or MEKK1-dependent phosphorylation can activate the purified IκBα kinase (Figure 4C).

The detailed molecular mechanism by which MEKK1 activates the IκBα kinase remains to be determined. One possibility is that MEKK1 inactivates a negative regulatory subunit of the IκBα kinase, just as cAMP binds to and induces the dissociation of the regulatory subunit of PKA (Francis and Corbin, 1994). Alternatively, MEKK1 may activate the catalytic subunit of the IκBα kinase that subsequently phosphorylates Ser-32 and -36 of IκBα. Yet another possibility is that MEKK1 initiates a MAPK-like cascade within the IκBα kinase complex, with the terminal kinase the subunit that phosphorylates Ser-32 and -36; this would be somewhat analogous to the organization of MAPK modules as high molecular weight complexes in yeast (Choi et al., 1994). The incorporation of ^{32}P into multiple subunits of the IκBα kinase complex in the presence of MEKK1Δ (Figure 7) could be consistent with any of these possibilities. It will be of interest to determine if the IκBα kinase subunit(s) targeted by MEKK1 is the same as those targeted by the ubiquitination system. Further conclusions must await the identification and characterization of subunits of the IκBα kinase.

Experimental Procedures

Plasmids

pCMV5-MEKK1 (which encodes the C-terminal 672 residues of MEKK1), pcDNA3-FlagMKK4, and pSRαHA-JNK1 were gifts of Dr. Roger Davis (University of Massachusetts, Worcester) and have been described (Derijard et al., 1994, 1995; Whitmarsh et al., 1995). pcDNA3-MEKK1 was constructed by subcloning the 2.4 kb EcoRI/EcoNI (blunt) fragment of pCMV5-MEKK1 encoding MEKK1 into the EcoRI/EcoRV site of pcDNA3. pcDNA3-FlagMEKK1Δ (K432M) consists of an N-terminal Flag epitope fused to the C-terminal 321 amino acid fragment of MEKK1 with the indicated mutation (amino acid numbering according to Lange-Carter et al., 1993) and was constructed by polymerase chain reaction (Ausubel et al., 1989). pcDNA3-FlagMEKK1Δ was constructed by replacing the 2.1 kb StuI fragment of pcDNA3-FlagMEKK1Δ (K432M), which encodes the C-terminal 262 amino acids with the corresponding fragment of pcDNA3-MEKK1. pCMV4-FlagIκBα and pCMV4-FlagIκBα(S32A/S36A) were gifts of Dr. Dean Ballard (Vanderbilt University) and have been described (Brockman et al., 1995). pcDNA1-cJun has been described (Du et al., 1993). pcDNA1-cJun(S63A/S73A) was constructed using overlapping polymerase chain reaction (Ausubel et al., 1989). pBS-IκBα, pBS-IκBα(S32A/S36A), pBS-FlagIκBα, pBS-FlagIκBα(S32A/S36A), $(PRDII)_2$CAT, $(PRDIV)_6$CAT, $(CRE)_6$CAT, −110IFN-βCAT, and pCMV-lacZ have been described (MacGregor and Caskey, 1989; Du and Maniatis, 1992; Thanos and Maniatis, 1992; Chen et al., 1995).

Tissue Culture and Transfection

HeLa and L929 cells were maintained in DME media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfections and virus infections, performed in 3.5 cm diameter wells, were conducted as described (Thanos and Maniatis, 1992). Cells were typically harvested at 41 to 49 hr posttransfection. CAT and β-galactosidase assays were performed as described (Sambrook et al., 1989). Protein concentrations were measured by the Bradford method.

Immunoprecipitations

Transfected IκBα

Cell pellets obtained by harvesting 3.5 cm diameter wells were lysed by the addition of 200 μl of Buffer A (20 mM Tris, pH 7.5, 0.4 M KCl, 4 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1% NP-40, 10% glycerol, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT), followed by three freeze/thaw cycles. After centrifugation at 14,000 × g for 5 min at 4°C, the supernatant (320 μg protein) was incubated with 20 μl of M2-agarose (IBI-Kodak) in 1 ml of Buffer A with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer A and once with 0.1X Buffer A.

^{35}S-Labeled FlagIκBα

In vitro–translated FlagIκBα was immunoprecipitated by incubation with 10 μl of M2-agarose in 1 ml of Buffer B (10 mM Tris, pH 7.6, 100 mM NaCl, 0.1% NP-40, 10 μg/ml leupeptin, 1 mM DTT) with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer B, once with Buffer C (10 mM Tris, pH 7.6, 1 mg/ml BSA, 10 μg/ml leupeptin, 1 mM DTT), and then eluted by the addition of 24 μl of Buffer C containing 0.7 mg/ml Flag peptide for 30 min on ice.

Western Blotting

Proteins were electrophoresed by SDS-PAGE and transferred to Immobilon-NC membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with rabbit anti-IκBα polyclonal antibodies (C21, Santa Cruz Biotechnology). Membranes were then incubated with goat anti-rabbit IgG-alkaline phosphatase or donkey anti-rabbit IgG-horseradish peroxidase conjugates, and developed using standard chromogenic or Enhanced Chemiluminescence (Amersham) substrates, respectively. Western blots of purified IκBα kinase employed antibodies (anti-MEKK1 [C22], anti-MKK4 [C20], anti-JNK1 [FL], anti-JNK2 [FL]) obtained from Santa Cruz Biotechnology.

Extract Preparation

HeLa S_3 cell cytoplasmic extracts were prepared by two methods. In the first ("rapid lysis procedure"), mid–logarithmic growth phase HeLa S_3 cells cultured in RPMI 1640 media supplemented with 5% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin were centrifuged at 2,600 × g for 10 min. Cells were resuspended in RPMI media containing 5% horse serum, and then either mock treated or incubated with 1000 U/ml TNF-α at 37°C. At various times, cells were centrifuged at 1,000 × g for 1 min at ambient temperature. Cells were then rapidly washed with ice-cold PBS, centrifuged again at 1,000 × g for 1 min, resuspended in ice-cold 50 mM Tris (pH 7.5), 1 mM EGTA, and then immediately lysed by dounce homogenization (15–20 strokes with an A-type pestle). The crude lysate was clarified by centrifugation at 4,600 × g for 10 min at 4°C, and the resulting supernatant immediately frozen at −80°C. In the second method ("S100"), HeLa S_3 cells were swollen in a hypotonic buffer and lysed, followed by removal of nuclei and centrifugation at 100,000 × g as described (Dignam et al., 1983). The supernatant was then dialyzed extensively against 20 mM Tris

(pH 7.5), 0.5 mM DTT. If not employed immediately, the extract was stored at −80°C.

Purification of IκBα Kinase

HeLa cell S100 cytoplasmic extract, prepared as above, was applied to a Mono-Q anion exchange column. The IκBα kinase activity was eluted with 0.2–0.3 M KCl in Buffer D (50 mM Tris, pH 7.5, 0.5 mM DTT), and then precipitated with 40% ammonium sulfate. The resuspended precipitates were dialyzed against 10 mM K_2HPO_4-KH_2PO_4 (pH 7.0), 0.5 mM DTT, and then applied to a hydroxylapatite column. After elution with 0.2 M K_2HPO_4-KH_2PO_4 (pH 7.0), the kinase-containing fractions were applied to a Superdex-200 gel filtration column equilibrated with 50 mM Tris (pH 7.5), 0.5 mM DTT, and 150 mM NaCl. The high molecular weight fractions that contained the kinase activity were applied to a Mono-Q column and eluted with a linear gradient of 150–325 mM NaCl in Buffer D. Fractions from the Superdex-200 and second Mono-Q chromatographies were assayed for IκBα kinase activity in the presence of ubiquitination components (Ubc4 and ubiquitin, in addition to E1 supplied by the wheat germ extract employed for in vitro translation of IκBα) (Chen et al., 1996), or recombinant MEKK1Δ.

Preparation of Recombinant Proteins

$(His)_6$MEKK1Δ and $(His)_6$MEKK1Δ (K432M) were purified using Ni-NTA agarose from Sf9 cells infected with baculovirus prepared using the Bac-to-Bac Expression System (GIBCO–BRL Life Technologies). pFastBacHT-MEKK1Δ and pFastBacHT-MEKK1Δ (K432M) were constructed by subcloning the 1.2 kb NcoI/XbaI coding sequence fragment of pcDNA3-FlagMEKK1Δ and pcDNA3-FlagMEKK1Δ (K432M), respectively, into the Nco I/Xba I site of pFastBacHTa. Recombinant bacmids and baculovirus were subsequently prepared according to the manufacturer's instructions. GST-MKK4 and GST-JNK1 were purified from E. coli HB101 transformed with pGEX-MKK4 and pGEX-JNK1, respectively, employing glutathione agarose affinity chromatography as described (Smith and Johnson, 1988). pGEX-MKK4 was constructed by subcloning the 1.1 kb BamHI (blunt)/Bsp120I (blunt) fragment of pcDNA3-FlagMKK4 containing the MKK4 coding sequence into the SmaI site of pGEX-3X (Pharmacia). pGEX-JNK1 was constructed by subcloning the 1.4 kb NcoI (blunt)/SalI fragment of pSRαHA-JNK1 containing the JNK1 coding sequence into the EcoRI (blunt)/SalI site of pGEX-5X-1 (Pharmacia). $(His)_6$IκBα was purified using Ni-NTA agarose from E. coli BL21(DE3)LysS transformed with pRSET-IκBα. pRSET-IκBα was constructed by subcloning the EagI (blunt)/HindIII fragment of pBS-IκBα containing the IκBα coding sequence into the PvuII/HindIII site of pRSET A (Invitrogen). The E2 enymes Ubc4 and GST-Ubc5 were prepared as described (Chen et al., 1996). Concentrations of recombinant proteins were determined by SDS-PAGE followed by staining with Coomassie blue and comparison with bovine serum albumin standards.

Protein Kinase Assays

Mobility Shift Assays

Typically, HeLa cell cytoplasmic extracts or purified IκBα kinase (from gel filtration chromatography as described above) was incubated with 0.5 μl of in vitro-translated, ^{35}S-labeled protein in a total volume of 10 μl containing 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, 10 mM phosphocreatine, 3.5 U/ml creatine phosphokinase, and 2.5 μM okadaic acid. In vitro-translated, ^{35}S-labeled IκBα, Flag-IκBα, and c-Jun, or their phosphorylation defective mutants, were prepared using TnT wheat germ extract kits (Promega) and pBS-IκBα, pBS-IκBα (S32A/S36A), pBS-FlagIκBα, pBS-FlagIκBα (S32A/S36A), pcDNA1-cJun, or pcDNA1-cJun(S63A/S73A) as templates.

[γ-^{32}P]ATP Labeling of IκBα

Enzyme was incubated with 0.5 μg $(His)_6$IκBα in 10 μl of 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2.5 μM okadaic acid, 200 μM ATP and 5 μCi of [γ-^{32}P]ATP. Incubations were carried out at 30°C for 30 min.

Dephosphorylation of IκBα Kinase Complex

Purified IκBα kinase (from gel filtration chromatography) was treated with MEKK1Δ in 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP for 30 min at 30°C. MEKK1Δ-activated IκBα kinase was separated from ATP by centrifugal gel filtration on Sephadex G50 and subsequently incubated with or without calf intestinal alkaline phosphatase (CIP) in 50 mM Tris (pH 7.8), 0.1 mM EDTA for 30 min at 30°C. IκBα kinase was then separated from CIP and MEKK1Δ by chromatography on a Superdex 200 column and assayed for IκBα kinase activity in the absence or presence of MEKK1Δ.

[γ-^{32}P]ATP Labeling of IκBα Kinase Complex

Two nanograms of MEKK1Δ was incubated in 7 μl of 70 mM Tris (pH 7.6), 7 mM $MgCl_2$, 3.5 μM okadaic acid, and 140 μM ATP for 15 min at 30°C. Subsequently, purified IκBα kinase (from the second Mono Q chromatography step as described above) and 10 μCi of [γ-^{32}P]ATP in a total volume of 3 μl were added and the incubation continued at 30°C for an additional 30 min. In control reactions, either MEKK1Δ or IκBα kinase was omitted.

Acknowledgments

Correspondence should be addressed to T. M. We thank Drs. Roger Davis and Dean Ballard for gifts of plasmids. We also thank members of the Maniatis laboratory for helpful discussions and critical readings of the manuscript. F. S. L. was supported by National Institutes of Health K08 grant AI01241 and by a Postdoctoral Research Fellowship for Physicians from the Howard Hughes Medical Institute. J. H. was supported by a postdoctoral fellowship from the American Cancer Society (PF-4053). This work was supported by National Institutes of Health grant AI20642 to T. M. and by ProScript, Inc.

Received October 11, 1996; revised November 25, 1996.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989). Current Protocols in Molecular Biology (New York: John Wiley and Sons, Inc.).

Bagrodia, S., Derijard, B., Davis, R.J., and Cerione, R.A. (1995). Cdc42 and PAK-mediated signaling leads to jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. *270*, 27995–27998.

Baldwin, A.S. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. *14*, 649–681.

Blank, J.L., Gerwins, P., Elliott, E.M., Sather, S., and Johnson, G.L. (1996). Molecular cloning of mitogen-activated protein/ERK kinase kinases (MEKK) 2 and 3. J. Biol. Chem. *271*, 5361–5368.

Brockman, J.A., Scherer, D.C., Hall, S.M., McKinsey, T.A., Qi, X., Lee, W. Y., and Ballard, D.W. (1995). Coupling of signal-response domain in IκB-α to multiple pathways for NF-κB activation. Mol. Cell. Biol. *15*, 2809–2818.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science *267*, 1485–1491.

Chen, Z.J., Hagler, J., Palombella, V., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκB-α to the ubiquitin-proteasome pathway. Genes and Dev. *9*, 1586–1597.

Chen, Z.J., Parent, L., and Maniatis, T. (1996). Site-specific phosphorylation of IκB-α by a novel ubiquitination-dependent protein kinase activity. Cell *84*, 853–862.

Choi, K.Y., Satterberg, B., Lyons, D.M., and Elion, E.A. (1994). Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell *78*, 499–512.

Coso, O.A., Chiarello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995). The small GTP-binding proteins rac1 and cdc42 regulate the activity of the JNK/SAPK pathway. Cell *81*, 1137–1146.

Derijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R.J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-ras that binds and phosphorylates the c-jun activation domain. Cell *76*, 1025–1037.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R.J., and Davis, R.J. (1995). Independent human MAP kinase signal

transduction pathways defined by MEK and MKK isoforms. Science *267*, 682–685.

Devary, Y., Rosette, C., DiDonato, J.A., and Karin, M. (1993). NF-κB activation by ultraviolet light not dependent on a nuclear signal. Science *261*, 1442–1445.

Diaz-Meco, M.T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M.M., Berra, E., Hay, R.T., Sturgill, T.W., and Moscat, J. (1994). ζPKC induces phosphorylation and inactivation of IκB-α in vitro. EMBO J. *13*, 2842–2848.

Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. *11*, 1475–1489.

Du, W., and Maniatis, T. (1992). An ATF/CREB binding site is required for virus induction of the human interferon β gene. Proc. Natl. Acad. Sci. USA *89*, 2150–2154.

Du, W., Thanos, D., and Maniatis, T. (1993). Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell *74*, 887–898.

Finco, T.S., and Baldwin, A.S. (1993). κB site-dependent induction of gene expression by diverse inducers of nuclear factor κB requires raf-1. J. Biol. Chem. *268*, 17676–17679.

Francis, S.H., and Corbin, J.D. (1994). Structure and function of cyclic nucleotide-dependent protein kinases. Annu. Rev. Physiol. *56*, 237–272.

Galcheva-Gargova, Z., Derijard, B., Wu., I.H., and Davis, R.J. (1994). An osmosensing signal transduction pathway in mammalian cells. Science *265*, 806–808.

Ghosh, S., and Baltimore, D. (1990). Activation of NF-κB by phosphorylation of its inhibitor IκB. Nature *344*, 678–682.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science *267*, 389–393.

Henkel, T., Machleidt, T., Alkalay, I., Kronke, M., Ben-Neriah, Y., and Baeuerle, P.A. (1993). Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature *365*, 182–185.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-jun activation domain. Genes and Dev. *7*, 2135–2148.

Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoue, J., and Ohno, S. (1996). MEK kinase is involved in tumor necrosis factor α-induced NF-κB activation and degradation of IκB-α. J. Biol. Chem. *271*, 13234–13238.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. *270*, 16483–16486.

Kyriakis, J.M., and Avruch, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. J. Biol. Chem. *271*, 24313–24316.

Kyriakis, J.M., Banerjee, P., Nikolaki, E., Dai, T., Ruble, E.A., Ahmad, M.F., Avruch, J., and Woodgett, J.R. (1994). The stress-activated protein kinase subfamily of c-jun kinases. Nature *369*, 156–160.

Lange-Carter, C.A., Pleiman, C.M., Gardner, A.M., Blumer, K.J., and Johnson, G.L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and raf. Science *260*, 315–319.

Liu, Z.-G., Hsu, H., Goeddel, D.V., and Karin, M. (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.

MacGregor, G.R., and Caskey, C.T. (1989). Construction of plasmids that express E. coli beta galactosidase in mammalian cells. Nucleic Acids Res. *17*, 2365.

Mellits, K.H., Hay, R.T., and Goodbourn, S. (1993). Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. *21*, 5059–5066.

Meyer, C.F., Wang, X., Chang, C., Templeton, D., and Tan, T.-H. (1996). Interaction between c-Rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating κB enhancer activation. J. Biol. Chem. *271*, 8971–8976.

Minden, A., Lin, A., Claret, F.-X., Abo, A., and Karin, M. (1995). Selective activation of the JNK-signaling cascade and c-Jun transcriptional activity by the small GTPases rac and cdc42hs. Cell *81*, 1147–1157.

Russell, M., Lange-Carter, C.A., and Johnson, G.L. (1995). Direct interaction between ras and the kinase domain of mitogen-activated protein kinase kinase kinase (MEKK1). J. Biol. Chem. *270*, 11757–11760.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sanchez, I., Hughes, R.T., Mayer, B.J., Yee, K., Woodgett, J.R., Avruch, J., Kyriakis, J.M., and Zon, L.I. (1994). Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-jun. Nature *372*, 794–798.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene *67*, 31–40.

Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. Cell *77*, 727–736.

Thanos, D., and Maniatis, T. (1992). The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell *71*, 777–789.

Thanos, D., Du, W., and Maniatis, T. (1993). The high mobility group protein HMG I(Y) is an essential structural component of a virus-inducible enhancer complex. Cold Spring Harbor Symp. Quant. Biol. *58*, 73–81.

Thevenin, C., Kim, S.J., Rieckmann, P., Fujiki, H., Norcross, M.A., Sporn, M.B., Fauci, A.S., and Kehrl, J.H. (1990). Induction of nuclear factor-κB and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific inhibitor of phosphatases 1 and 2A. New Biol. *2*, 793–800.

Traenckner, E.B.-M., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S., and Baeuerle, P.A. (1995). Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. *14*, 2876–2883.

Whitmarsh, A.J., Shore, P., Sharrocks, A.D., and Davis, R.J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. Science *269*, 403–407.

Xu, S., Robbins, D.J., Christerson, L.B., English, J.M., Vanderbilt, C.A., and Cobb, M. (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.

Yan, M., Dai, T., Deak, J.C., Kyriakis, J.M., Zon, L.I., Woodgett, J.R., and Templeton, D.J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature *372*, 798–800.

Yang, Y.L., Reis, L.F.L., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B.R.G., Aguet, M., and Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J. *14*, 6095–6106.