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Immunology

NF-κB-inducing kinase activates IKK-α by phosphorylation of Ser-176

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ABSTRACT **Activation of the transcription factor NF-κB by inflammatory cytokines involves the successive action of NF-κB-inducing kinase (NIK) and two IκB kinases, IKK-α and IKK-β. Here we show that NIK preferentially phosphorylates IKK-α over IKK-β, leading to the activation of IKK-α kinase activity. This phosphorylation of IKK-α occurs specifically on Ser-176 in the activation loop between kinase subdomains VII and VIII. A mutant form of IKK-α containing alanine at residue 176 cannot be phosphorylated or activated by NIK and acts as a dominant negative inhibitor of interleukin 1- and tumor necrosis factor-induced NF-κB activation. Conversely, a mutant form of IKK-α containing glutamic acid at residue 176 is constitutively active. Thus, the phosphorylation of IKK-α on Ser-176 by NIK may be required for cytokine-mediated NF-κB activation.**

Many of the common proinflammatory properties of tumor necrosis factor (TNF) and interleukin 1 (IL-1) are mediated by the transcription factor NF-κB (1–3). Under normal conditions, NF-κB exists in a cytoplasmic complex with an inhibitor protein IκB (1–3). The activation of NF-κB requires phosphorylation of IκB-α at Ser-32 and Ser-36 (4). This phosphorylation targets IκB-α for ubiquitination and proteosome-mediated degradation, thereby releasing NF-κB to enter the nucleus and activate a series of genes involved in the inflammatory response (5).

It is now known that NF-κB activation by TNF and IL-1 involves signal transduction cascades containing several intermediate signaling proteins. TNF initiates its signaling by binding to and trimerizing the type 1 TNF receptor, TNF-R1 (6, 7). Several cytoplasmic proteins, including TNF-R1-associated death domain protein (TRADD) (6), TNF receptor-associated factor (TRAF2) (8), and receptor-interacting protein (RIP) (9), are then recruited to the intracellular domain of TNF-R1 where they form an active signaling complex. Overexpression of each of these proteins can activate the signaling cascade leading to NF-κB activation. On the other hand, IL-1 induces the formation of a complex including two distinct receptor chains, IL-1RI and IL-1RAcP (10), the adaptor protein MyD88 (11), and the protein kinase IRAK (12). Following its activation, IL-1 receptor-associated kinase (IRAK) is released from the receptor complex (11) and associates with TRAF6 (13).

These distinct TNF and IL-1 pathways merge at the level of the protein kinase NF-κB-inducing kinase (NIK) (14). NIK, which is a member of the mitogen-activating protein (MAP) kinase kinase kinase (MAP3K) family, was originally identified as a TRAF2-interacting protein. NIK activates NF-κB when overexpressed, and kinase-inactive mutants of NIK behave as dominant-negative inhibitors that suppress NF-κB activation mediated by TNF, IL-1, TRADD, RIP, TRAF2, TRAF5, and TRAF6 (14, 15). Thus, NIK is a common mediator in the NF-κB signaling cascades triggered by TNF and IL-1 that acts downstream of the receptor complexes. However, NIK is not involved in TNF and IL-1-stimulated kinase pathways that lead to the activation of the Jun N-terminal kinase (15). The details of the molecular mechanism(s) by which NIK itself becomes activated are not yet understood.

In an effort to identify downstream targets of NIK, conserved helix–loop–helix ubiquitous kinase (CHUK) was isolated in a yeast two-hybrid screen (16). CHUK was also biochemically purified from TNF-treated HeLa cells by DiDonato *et al.* (17) and by Mercurio *et al.* (18). CHUK is an 85-kDa protein kinase of previously unknown function (19). CHUK directly associates with, and specifically phosphorylates IκB-α on Ser-32 and -36 (16, 17). A catalytically inactive mutant of CHUK is a dominant-negative inhibitor of TNF-, IL-1, TRAF2-, TRAF6-, and NIK-induced NF-κB activation and CHUK kinase activity is stimulated by cytokine treatment (16, 17). Based on these results, CHUK has been redesignated as IκB kinase-α (IKK-α). Cells cotransfected with NIK and IKK-α display elevated IKK-α activity (16). A second IKK, IKK-β, with 52% amino acid identity to IKK-α, was also recently identified (18, 20, 21). IKK-α and IKK-β exist in a heterocomplex form that is able to interact with NIK (20). Thus, IKK-α and IKK-β may both represent downstream targets of NIK, and all three of these kinases are likely present in a large IκB kinase complex of 700–900 kDa (17, 20, 22).

Because NF-κB can be activated by numerous stimuli, the IKK complex may serve as an integration point for signals emanating from many different pathways. Although the molecular mechanism for activation of this kinase complex is not known, IKK-α is likely a downstream target of NIK, because NIK coexpression stimulates the ability of IKK-α to phosphorylate IκB-α. Additionally, a dominant negative form of IKK-α blocks NIK-induced NF-κB activation (16, 20). Because NIK is a member of the MAP3K family, it may activate downstream kinases by specific phosphorylation events similar to other MAP3K family members. In this study, we report that IKK-α is a better substrate than IKK-β for phosphorylation by NIK. The primary site of IKK-α phosphorylation by NIK is Ser-176 in the kinase activation loop. Phosphorylation of this residue correlates with activation of IKK-α.

MATERIALS AND METHODS

Cell Culture and Biological Reagents. Recombinant human TNF and IL-1 were provided by Genentech, Inc. (South San Francisco). The anti-FLAG mAb M2 affinity resin and purified FLAG peptide were purchased from Eastman Kodak. Rabbit anti-FLAG and anti-Myc polyclonal antibodies were from Santa Cruz Biotechnology. Human embryonic kidney

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Abbreviations: NIK, NF-κB-inducing kinase; IKK, IκB kinase; TNF, tumor necrosis factor; IL, interleukin; MAP, mitogen-activating protein; MAP3K, MAP kinase kinase kinase; β-gal, β-galactosidase.

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293 cells, 293/IL-1RI cells (12) and HeLa cells were maintained as described (6).

Expression Vectors. Mammalian cell expression vectors encoding wild-type and kinase-inactive versions of NIK, IKK-α, and IKK-β have been described (15, 16, 20). IKK-α(KA) and IKK-β(KA) signify lysine to alanine changes at amino acid 44 of IKK-α and IKK-β, respectively. NIK(KA) represents lysine to alanine changes at amino acids 429 and 430 of NIK. The control expression plasmid pRK5, the NF-κB-dependent E-selectin-luciferase reporter gene plasmid and plasmid RSV-β-galactosidase were also described (23, 24). Expression vectors encoding IKK-α and IKK-β mutants with alanine or glutamic acid replacing serine and threonine residues in the activation loop of IKK-α and IKK-β [IKK-α(S176A), IKK-α(KA)S176A, IKK-α(S176E), IKK-α(T179A), IKK-α(KA)T179A, IKK-α(S180A), IKK-α(KA)S180A, IKK-β(S177A)] were constructed using Stratagene Quickchange site-directed mutagenesis kit. All the mutations were verified by DNA sequencing analysis.

Immunoprecipitation, Western Blot Analysis and *in Vitro* Kinase Assays. 293 cells or HeLa cells were transiently transfected with expression plasmids by using calcium phosphate as described (6). Between 24–36 hr later, cells were washed with cold PBS and lysed in Nonidet P-40 lysis buffer containing 50 mM Hepes (pH 7.6), 250 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, and Complete protease inhibitors (Boehringer Mannheim) (16, 20). Cell lysates were cleared and incubated for 2–4 hr at 4°C with anti-FLAG M2 antibody resin (Kodak), washed extensively with lysis buffer and eluted with FLAG peptide (300 μg/ml, Kodak) or not eluted. *In vitro* kinase assays were performed with eluted proteins or immune complexes and bacterially synthesized IκB-α (amino acids 1–250) proteins (16) in 20 μl kinase buffer containing 20 mM Tris·HCl (pH7.6), 10 mM MgCl2, 0.5 mM DTT, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) at room temperature for 30 min (16). Samples were analyzed by 10% SDS/PAGE and autoradiography. Immunoblotting analyses were performed with rabbit polyclonal antibodies and detected by alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody.

Reporter Assays. For reporter gene assays, 293 cells, 293/IL-1RI cells, or HeLa cells were seeded into six-well plates. Cells were transfected the following day by the calcium phosphate precipitation method with 0.5 μg E-selectin-luciferase reporter gene plasmid, 1 μg pRSV-β-gal plasmid, and various amounts of each expression construct. The total DNA transfected (4.5 μg) was kept constant by supplementation with the control vector pRK5. In the NIK and IKK-α synergy experiments, 0.01 μg of NIK, IKK-α(WT), and IKK-α(S176A) were used for each 35-mm well. After 24 hr, cells were either left untreated or stimulated with IL-1 (10 ng/ml), or TNF (100 ng/ml) for 5 hr prior to harvest. Reporter gene activity was determined with the Luciferase Assay System (Promega). The results were normalized for transfection efficiency on the basis of β-gal expression.

RESULTS

***In Vitro* Phosphorylation of IKK-α by NIK.** NIK directly interacts with IKK-α and IKK-β, and the phosphorylation of IκB-α by IKK-α and IKK-β is enhanced by NIK coexpression (16, 20). These results suggest that IKK-α and IKK-β may be NIK-activated IκB-α kinases that link TNF-and IL-1-induced kinase cascades to NF-κB activation. To investigate if IKK-α and IKK-β can be phosphorylated by NIK, we transiently expressed FLAG epitope-tagged wild-type or kinase-inactive mutants of NIK, IKK-α, and IKK-β in human embryonic kidney 293 cells (Fig. 1). The epitope-tagged proteins were immunoprecipitated with an anti-FLAG antibody, and incubated with [γ-^{32}P]ATP. In these assays, wild-type IKK-α, IKK-β, and NIK become autophosphorylated when expressed individually, while mutants of all three kinases containing lysine-to-alanine (KA) substitutions in their ATP-binding sites were not autophosphorylated (16). The IKK-α(KA) and IKK-

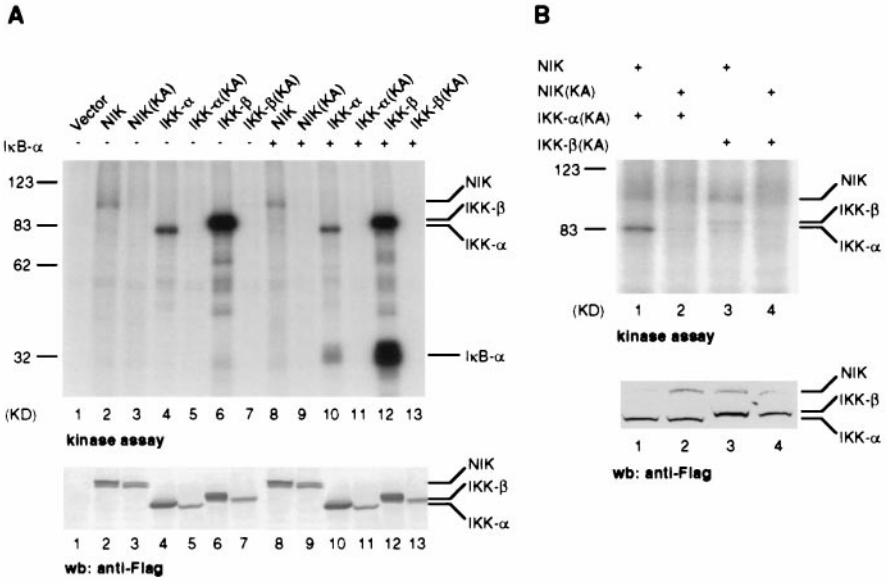

FIG. 1. In vitro phosphorylation of IKK-α by NIK. (*A*) Autophosphorylation and phosphorylation of IκB-α by various kinases. 293 cells were transiently transfected with expression plasmids encoding FLAG epitope-tagged wild-type or KA mutants of NIK, IKK-α and IKK-β. Thirty-six hours after transfection, extracts were immunoprecipitated with anti-FLAG mAb affinity resin and FLAG-tagged proteins were purified as described in *Materials and Methods*. Purified proteins were incubated with [γ-^{32}P]ATP in the presence or absence or bacterially synthesized protein IκB-α (amino acids 1–250), resolved by SDS/PAGE, and analyzed by autoradiography. The amounts of proteins used in the reactions were determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*). The positions of IKK-α, IKK-β, and NIK are indicated. (*B*) Phosphorylation of IKK-α(KA) and IKK-β(KA) by NIK. 293 cells were transiently transfected with expression plasmids encoding FLAG epitope-tagged wild-type NIK, IKK-α(KA), or IKK-β(KA). Purified proteins were incubated with [γ-^{32}P]ATP, resolved by SDS/PAGE, and analyzed by autoradiography. The amounts of proteins used in the reactions were determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*). The positions of IKK-α, IKK-β, and NIK are indicated.

β(KA) mutants were also unable to phosphorylate IκB-α (Fig. 1*A*).

To examine its ability to phosphorylate IKK-α and IKK-β, we coexpressed NIK with the catalytically inactive IKK mutants. We found that NIK can phosphorylate IKK-α(KA), but only weakly phosphorylates IKK-β(KA) (Fig. 1*B*, compare lanes 1 and 3). The kinase-inactive NIK does not phosphorylate IKK-α(KA) or IKK-β(KA) (Fig. 1*B*, lanes 2 and 4). In addition, we found that purified, baculovirus-expressed IKK-α(KA) can be phosphorylated by baculovirus-expressed NIK, but not by baculovirus-expressed NIK(KA) (data not shown).

Ser-176 of IKK-α Is Phosphorylated by NIK. Because NIK is a MAP3K-related kinase, it may activate a downstream kinase or kinases in a manner similar to other members of the MAP3K family. MAP3Ks activate MAP2Ks (such as MEK1) by phosphorylating serine and threonine residues in the "activation loop" between kinase subdomains VII and VIII (25–27). Therefore, we examined serine and threonine residues in the activation loop of IKK-α as we have no evidence for IKK-α being tyrosine-phosphorylated (unpublished data).

There are two serines (residues 176 and 180) and a threonine (residue 179) in the activation loop of IKK-α (Fig. 2). To test if Ser-176, Thr-179, or Ser-180 are phosphorylated by NIK, each of these three residues was mutated to alanine in the background of the inactive IKK-α(KA) mutant to eliminate IKK-α autophosphorylation activity, and tested for phosphorylation by NIK. Mutation of Ser-176 to alanine [IKK-α(S176A)] significantly reduced the phosphorylation of IKK-α by NIK, while the T179A and S180A mutants were still efficiently phosphorylated (Fig. 3). These results indicate that Ser-176 represents the major site of IKK-α phosphorylation by NIK.

Loss of Activation of IKK-α(S176A) by NIK. Because NIK can phosphorylate Ser-176 in the activation loop of IKK-α and stimulate IκB-α phosphorylation by IKK-α, it is possible that Ser-176 phosphorylation may be required for IKK-α activity. If so, the phosphorylation of IκB-α by IKK-α should be greatly impaired when Ser-176 of wild-type IKK-α is mutated to alanine. IKK-α(S176A) was expressed, purified, and found to have greatly reduced activity as measured by both its autophosphorylation and its ability to phosphorylate IκB-α (Fig. 4*A*). In contrast, mutation of the equivalent serine in IKK-β results in a kinase, IKK-β(S177A), that is fully active in autophosphorylation and in phosphorylation of IκB-α (Fig. 4*B*).

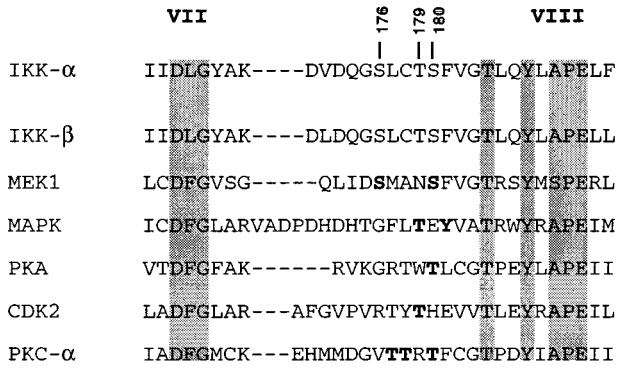

FIG. 2. Alignment of IKK-α amino acid sequences with several kinases in the activation loop region. The D(F/L)G and (A/S)PE residues that are characteristic of kinase subdomains VII and VIII are shaded. The conserved threonine and tyrosine residues in the TXXY motif adjacent to subdomain VIII are also shaded. The activating phosphorylation sites in MEK1 (25, 26), MAPK (30), PKA (31), CDK2 (32), and PKC-α (33) are shown in boldface. The position of the serine and threonines residues of IKK-α are indicated. The sequence of the activation loop of IKK-β is also included.

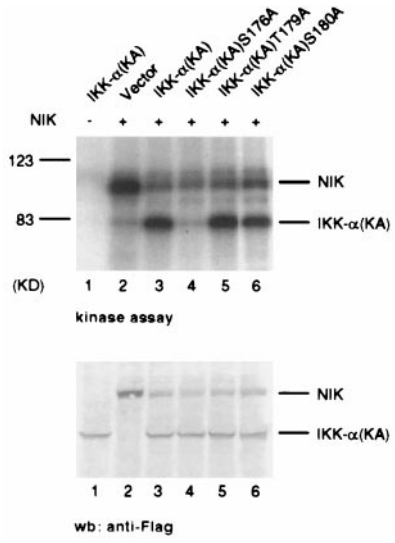

FIG. 3. Ser-176 in the activation loop of IKK-α is a major site of phosphorylation by NIK. Individual serine and threonine residues in the activation loop of IKK-α kinase domain were mutated to alanine. Each IKK-α mutant protein also contained the KA mutation in the ATP-binding site to prevent autophosphorylation. 293 cells were transiently transfected with expression plasmids encoding the indicated FLAG epitope-tagged proteins. Thirty-six hours after transfection, immunopurified proteins were incubated with [γ-^{32}P]ATP, resolved by SDS/PAGE, and analyzed by autoradiography. The amount of protein used in each reaction was determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*).

To determine whether IKK-α(S176A) is also defective in NF-κB activation, we compared IKK-α and IKK-α(S176A) in an NF-κB reporter gene assay in transiently transfected HeLa cells. As expected (16), expression of IKK-α modestly activated the NF-κB luciferase reporter gene in a dose-dependent manner. Mutation of Ser-176 to alanine abrogated the ability of IKK-α to activate NF-κB, similar to the IKK-α(KA) mutant (Fig. 4*C*).

If phosphorylation of Ser-176 is required to activate IKK-α, then mutation of this site should impair the ability of IKK-α to be activated by NIK. To test this, we coexpressed either FLAG epitope-tagged IKK-α or IKK-α(S176A) with Myc epitope-tagged wild-type NIK. We then specifically immunopurified the FLAG epitope-tagged IKK-α proteins and assayed them for IκB-α phosphorylation activity in an *in vitro* kinase assay. The phosphorylation of IκB-α by IKK-α was significantly enhanced when IKK-α was stimulated by NIK, but NIK failed to activate IKK-α(S176A) kinase activity to a similar extent (Fig. 5*A*).

The inability of NIK to activate IKK-α(S176A) is not only reflected by *in vitro* kinase assay but is also observed in tissue culture cells by using an NF-κB reporter gene assay. As shown in Fig. 5*B*, low levels of NIK and IKK-α synergistically activated the NF-κB luciferase reporter gene when coexpressed. This synergy was not observed when NIK is coexpressed with IKK-α(S176A).

IKK-α(S176A) Is a Dominant Negative Inhibitor of IL-1- and TNF-Induced NF-κB Activation. IKK-α associates with both NIK and IKK-β (16, 20). Because the IKK-α(S176A) mutant is inactive in both kinase and NF-κB reporter assays, it might compete with endogenous IKK-α for binding to NIK, IKK-β, or IκB-α and thereby block the activation of NF-κB. To test this, we determined the effect of IKK-α(S176A) on IL-1- and TNF-induced NF-κB activation in reporter gene assays in 293/IL-1RI cells. As shown in Fig. 6, overexpression of IKK-α(S176A) blocked both IL-1- and TNF-induced reporter gene activation in a dose-dependent manner. In addition, overexpression of IKK-α(S176A) blocked NIK-, TRAF2-, and

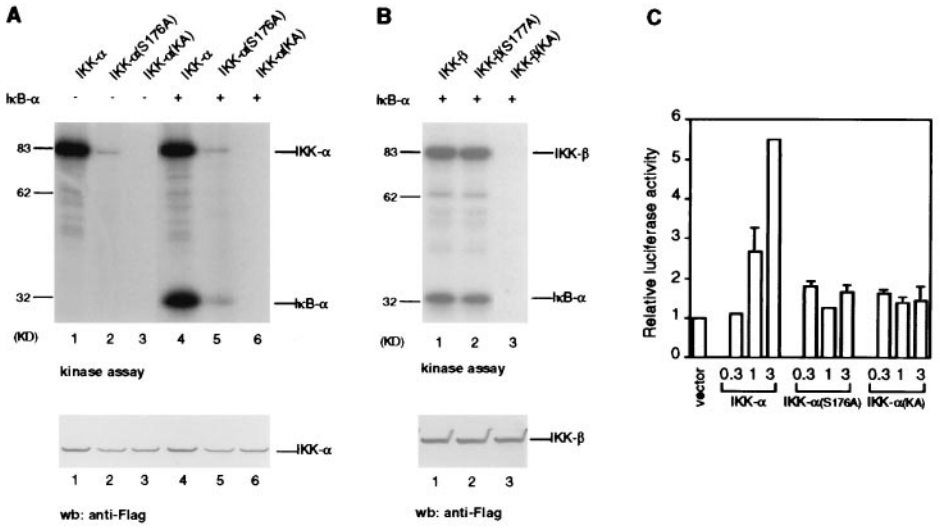

FIG. 4. IKK-α(S176A) has reduced kinase activity and NF-κB activation. (*A*) IKK-α(S176A) has reduced kinase activity. 293 cells were transiently transfected with the indicated epitope-tagged expression vectors. Thirty-six hours after transfection, IKK-α proteins were immunopurified with anti-FLAG mAb affinity resin and used in *in vitro* kinase reactions with IκB-α and [γ-^{32}P]ATP. (*Lower*) The protein expression in each lane is shown. (*B*) IKK-β(S177A) has similar kinase activity as IKK-β. 293 cells were transiently transfected with the indicated epitope-tagged expression vectors. Thirty-six hours after transfection, IKK-β proteins were immunopurified with anti-FLAG mAb affinity resin and used in *in vitro* kinase reactions with IκB-α and [γ-^{32}P]ATP. (*Lower*) The protein expression in each lane is shown. (*C*) IKK-α(S176A) is defective in NF-κB activation. HeLa cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α expression vector as indicated. Twenty-four hours after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

TRAF6-induced reporter gene activation in a dose-dependent manner (data not shown).

Mutation of Ser-176 to Glutamic Acid Activates IKK-α. Phosphorylation of IKK-α at Ser-176 introduces negative charge into this portion of the protein and results in kinase activation. It has been shown that substitution with negatively charged amino acids in the activation loop of other kinases can mimic activation (26, 27). Therefore we constructed an IKK-α(S176E) mutant, which contains glutamic acid at position 176. We expressed different doses of IKK-α or IKK-α(S176E) in HeLa cells, and measured the ability of immunopurified IKK-α or IKK-α(S176E) to phosphorylate IκB-α. At equivalent levels of expression, IKK-α(S176E) had significantly greater kinase activity than IKK-α, as measured by either autophosphorylation or phosphorylation of IκB-α (Fig. 7*A*). We also compared IKK-α and IKK-α(S176E) in an NF-κB reporter gene assay in transiently transfected HeLa cells. Mutation of Ser-176 to glutamic acid significantly enhanced the ability of IKK-α to activate NF-κB (Fig. 7*B*).

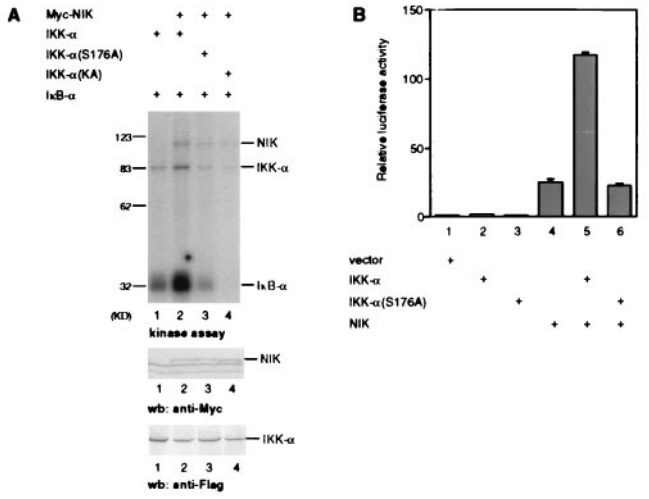

FIG. 5. Loss of IKK-α(S176A) activation by NIK. (*A*) Loss of IKK-α(S176A) activation by NIK in kinase assay. 293 cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176A) and Myc-epitope-tagged NIK. IKK-α proteins (and coprecipitating Myc-NIK proteins) were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out by using bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of protein used were determined by immunoblotting with anti-Myc polyclonal antibodies (*Middle*), and with anti-FLAG polyclonal antibodies (*Bottom*). (*B*) Loss of IKK-α(S176A) activation by NIK in an NF-κB reporter gene assay. 293 cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α and NIK expression vectors as indicated. Thirty to 36 hr after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

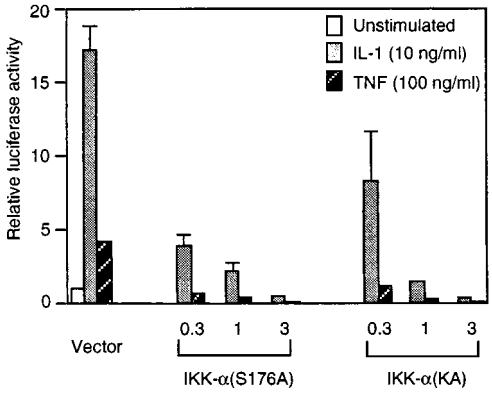

FIG. 6. IKK-α(S176A) is a dominant negative inhibitor of IL-1 and TNF-induced NF-κB activation. 293/IL-1RI cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α(S176A) expression vector as indicated. Twenty-four hours after transfection, cells were either left untreated, or stimulated for 6 hr with IL-1 (10 ng/ml) or TNF (100 ng/ml) prior to harvest. Luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

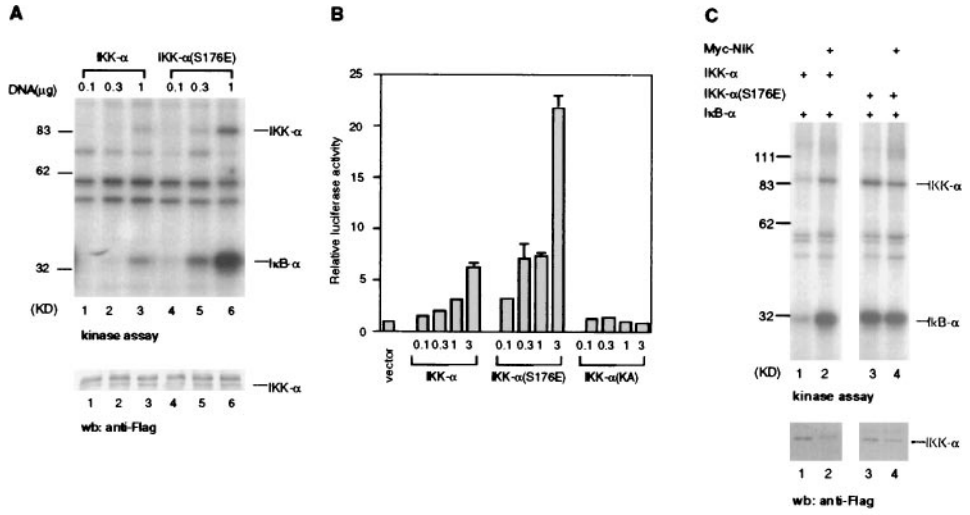

FIG. 7. IKK-α(S176E) is constitutively active. (*A*) IKK-α(S176E) has significantly greater activity than IKK-α in kinase assay. HeLa cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176E) at different doses. Thirty hours later, IKK-α proteins were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out with bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of protein used were determined by immunoblotting with anti-FLAG antibodies (as shown in the lower panel). (*B*) IKK-α(S176E) has significantly greater activity than IKK-α in an NF-κB reporter gene assay. HeLa cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α expression vector as indicated. Twenty-four hours after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages ($\pm$SEM) of duplicate samples for one representative experiment. (*C*) IKK-α(S176E) activity is independent of NIK activation. HeLa cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176E) and Myc-epitope-tagged NIK. IKK-α proteins were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out by using bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of IKK-α protein used were determined by immunoblotting with anti-FLAG antibodies (*Lower*).

The IKK-α(S176E) mutant is more active than wild-type IKK-α in both kinase assay and reporter assay. However, it is still important to know whether the IKK-α(S176E) mutant can be further activated by the upstream kinase NIK. In the experiments shown in Fig. 7*C*, we expressed FLAG epitope-tagged wild-type IKK-α or the IKK-α(S176E) mutant together with Myc epitope-tagged NIK in HeLa cells. We then measured the ability of the immunopurified IKK-α to phosphorylate recombinant IκB-α. The activity of wild-type IKK-α was strongly enhanced upon activation by the coexpressed NIK kinase. In contrast, IKK-α(S176E) mutant was similarly active when expressed either with or without NIK, suggesting that IKK-α(S176E) activity is independent of upstream activation.

DISCUSSION

The TNF- and IL-1-induced NF-κB activation pathways converge at NIK, a MAP3K-related serine/threonine kinase (14). NIK forms a complex with two IKKs, IKK-α and IKK-β (16, 20). IKK-α and IKK-β are serine/threonine kinases that phosphorylate members of the IκB family on two specific serine residues in a signal-induced process that is required for IκB degradation and NF-κB activation (16–18, 20, 21). In this study, we have shown that IKK-α is activated via serine phosphorylation by the upstream kinase NIK. NIK phosphorylates IKK-α on Ser-176 that lies in the activation loop (28) between subdomains VII and VIII. Mutation of Ser-176 to alanine results in a kinase-inactive form of IKK-α that is impaired not only in phosphorylating IκB-α and activating an NF-κB reporter gene, but which also can no longer be activated by NIK. Conversely, replacement of Ser-176 with glutamic acid results in a constitutively active IKK-α whose activity in both kinase and NF-κB reporter assays is independent of upstream kinase NIK. The importance of Ser-176 is further suggested by the ability of the IKK-α(S176A) mutant to block IL-1- and TNF-induced NF-κB activation. Although IKK-α can be activated via NIK-mediated phosphorylation on Ser-176, it is possible that other kinases may exist that can also phosphorylate and activate IKK-α.

The site of the NIK-activating phosphorylation of IKK-α lies between kinase subdomains VII and VIII. This phosphorylation and activation of IKK-α by NIK is reminiscent of the MAP kinase pathway in which the upstream kinases Raf and MEKK activate MEK via phosphorylation on Ser-218 and Ser-222 between kinase subdomains VII and VIII (25–27). MEK then activates MAPK by phosphorylating its corresponding activation loop on Thr-183 and Tyr-185 (30). The crystal structure of MAP kinase demonstrates that the activation loop lies in a solvent-exposed portion of the protein and phosphorylation of residues in this region can stabilize the kinase in an active conformation (28, 29). The spatial conservation of the sites activating kinase activity suggests that this mode of regulation is strongly conserved, especially among kinases found in signal transduction cascades. Activation sites in other related kinases might thus be inferred by homology to this region.

Although IKK-α and IKK-β share 52% identity, they are differentially phosphorylated by NIK. NIK appears to phosphorylate IKK-α better than IKK-β. However, we do not know if this difference is reflected in biological differences in the signaling processes affected by these two kinases. Recent experiments suggest that IKK-α and IKK-β form a heterocomplex that interacts directly with the upstream kinase NIK (18, 20, 21). IKK-β may be an inherently better IκB-α kinase than IKK-α and therefore might not need to be further activated by NIK phosphorylation. It is also possible IKK-β may become activated by autophosphorylation when overexpressed. Alternatively, IKK-β may also require phosphorylation by IKK-α or another kinase to be activated. Mercurio *et al.* (16) recently reported that an IKK-β(SS177, 181EE) mutant is constitutively active, supporting the view that phosphorylation also plays an important role in the activation of IKK-β.

IKK-α can be activated by a variety of external stimuli (17). Although the activation mechanism of IKK-α is emerging, little is known about the IKK-α inactivation process that occurs rapidly following the activation (17). It is possible that a phosphatase specifically dephosphorylates Ser-176 and inac-

tivates IKK-α, or that additional sites on IKK-α become dephosphorylated, resulting in enzyme inactivation.

We can now fill in additional details to the pathway by which TNF binding on the cell surface results in NF-κB activation in the nucleus. TNF interaction with TNF-R1 results in receptor trimerization and subsequent association with the adaptor molecule TRADD via the death domains of both proteins. TRADD then recruits TRAF2, RIP, and other signaling molecules, resulting in the formation of the TNF-R1 signaling complex. In a step that is not yet understood and that may require RIP and/or TRAF proteins, NIK becomes activated. NIK then phosphorylates IKK-α (or the α subunit of an IKK-α/IKK-β heterodimer) on Ser-176 in the IKK-α activation loop. Once activated by NIK, IKK-α phosphorylates Ser-32 and Ser-36 of IκB-α, signaling IκB-α for degradation, and allowing NF-κB translocation to the nucleus.

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