Negative Regulation of the Nuclear Factor κB-inducing Kinase by a cis-Acting Domain*

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Nuclear factor κB (NF-κB)-inducing kinase (NIK) participates in the activation of NF-κB, a family of eukaryotic transcription factors that mediate cell growth and transformation. NIK activates the IκB kinase both in vivo and in vitro, although how the activity of NIK is regulated has remained unclear. Here we show that the N-terminal region of NIK contains a negative-regulatory domain (NRD), which is composed of a basic motif and a proline-rich repeat motif. Deletion of these motifs leads to a marked enhancement of NIK function. We further demonstrate that the N-terminal NRD interacts with the C-terminal region of NIK, thereby inhibiting the binding of NIK to its substrate IκB kinase. Consistently, when expressed alone, the NRD potently inhibits NIK-mediated NF-κB signaling. These results provide a new insight into the mechanism of NIK regulation.

Nuclear factor κB (NF-κB)† represents a family of eukaryotic transcription factors participating in regulation of immune response, cell growth, and survival (reviewed in Refs. 1–3). The NF-κB factors are normally sequestered in the cytoplasmic compartment by physical association with inhibitors, including IκBα and related proteins (4). In response to diverse stimuli, including cytokines, mitogens, and certain viral gene products, IκBα is rapidly phosphorylated and degraded, which allows the liberated NF-κB to translocate to the nucleus and participate in target gene transactivation (5–7). Recent molecular cloning studies have identified a multisubunit IκB kinase, which mediates the signal-induced phosphorylation of IκBα (8). The IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ (9). The catalytic activity of both IKKα and IKKβ can be activated by different NF-κB inducers, including the inflammatory cytokines, tumor necrosis factor α and interleukin-1(10–14), and the T cell receptor and CD28 costimulatory signals of T cell activation (15).

Although precisely how IKKs respond to the various stimuli remains unclear, recent studies have identified potential upstream kinases, such as the NF-κB-inducing kinase (NIK) and the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1) (16–18), both belonging to the mitogen-activated protein kinase kinase kinase (MAP3K) family. NIK was originally identified as a protein interacting with the TRAF-2 component of the tumor necrosis factor α receptor complex (19). This MAP3K physically interacts with IKKα and IKKβ and stimulates the catalytic activity of these IKKs (10, 11). NIK interacts with IKKs via its C-terminal region, and this interaction appears to be required for its function in NF-κB signaling (20). When expressed in mammalian cells, NIK also form homodimers or oligomers (20).

Materials and Methods

Plasmid Constructs—pCMV4–1HA is a modified form of the pCMV4 mammalian expression vector (21). It carries one copy of the influenza hemagglutinin (HA) epitope tag (YPYDVPDYA) together with restriction sites for in-frame cloning of cDNA inserts. The expression vectors encoding wild type and truncated forms of NIK were generated by PCR amplification of a human NIK cDNA (kindly provided by Dr. David Wallach; Ref. 19), followed by subcloning the amplified DNA fragments into the pCMV4–1HA vector. The NIK truncation mutants are designated by the specific amino acid residues retained in the mutant protein. For example, NIK(33–947) contains the region from amino acid 33 to amino acid 947. The internal deletion mutants of NIK were generated by site-directed mutagenesis (Stratagene) using the wild type NIK expression vector as template. The Myc-tagged NIK(1–386) was obtained from Dr. Warner Greene (20). The dominant-active MEKK1 was provided by Dr. Michael Karin. The expression vectors for IKKα, IKKβ, and IκBα, and the κB-TATA-luc reporter plasmid were described previously (22).

Immunoblotting and Immunoprecipitation Assays—Human 293 kidney carcinoma cells were seeded in 0.1% gelatin-treated 24-well plates (2.5 × 10^4 cells/well) and transfected using DEAE-dextran (23) with 0.1 μg of HA-IκBα and other indicated expression vectors. After 40 h, whole cell extracts were prepared and analyzed by immunoblotting as described previously (24) using anti-HA antibody.

For immunoprecipitation studies, the cells were transfected in six-well plates and lysed in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.01 volume of a protease inhibitor mixture (Ref. 24)). Whole cell lysates were subjected to immunoprecipitation in the radioimmune precipitation buffer as described previously (24), and the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting.

Luciferase Assays—Jurkat T cells (1.25 × 10^6) were transfected using DEAE-dextran (23) with 50 ng of κB-TATA-luc together with the indicated cDNA expression vectors. After 40 h, the cells were lysed in 120 μl of reporter lysis buffer (Promega, luciferase reporter system). Luciferase activity was detected by mixing 5 μl of cell extracts with 25 μl of...
RESULTS

The C-terminal Non-catalytic Region of NIK Is Required for Its Signaling Function—Transient transfection assays have demonstrated that NIK stimulates the catalytic activity of IKKα and IKKβ, leading to in vivo phosphorylation of IkBα (10, 11). Since the phosphorylated IkBα migrates more slowly on SDS gels (25), it can be easily detected by immunoblotting assays. This in vivo assay approach was used to determine the functional domains of NIK. Briefly, wild type or mutant forms of NIK were coexpressed with IKKα or IKKβ in 293 cells together with the substrate protein IkBα. In the absence of NIK, neither IKKα (Fig. 1B, lane 2) nor IKKβ (lane 10) exhibited appreciable kinase activity, since the coexpressed IkBα was not phosphorylated. However, when the IKKs were expressed together with wild type NIK, IkBα was efficiently phosphorylated, as demonstrated by the appearance of the more slowly migrating IkBα band (Fig. 1B, lanes 3 and 11).

Luciferase assay approach was used to determine the expression level of transfected NIK and NIK mutants. The HA-tagged NIK was not phosphorylated. However, when the IKKs were expressed together with wild type NIK, IkBα was efficiently phosphorylated, as demonstrated by the appearance of the more slowly migrating IkBα band (Fig. 1B, lanes 3 and 11).

Fig. 1. Determination of C-terminal sequences of NIK required for its function in the induction of IkBα phosphorylation. 
A, schematic summary of C-terminal truncation mutants of NIK. The catalytic domain is shown in black boxes. B, IkBα phosphorylation in 293 cells induced by IKKs and NIK. 293 cells were transfected (in 24-well plates) with HA-tagged IkBα (0.1 μg), HA-tagged wild type (WT) or mutant forms of NIK (50 ng), together with either IKKα (25 ng, lanes 2–8) or IKKβ (10 ng, lanes 10–16). Whole cell extracts were subjected to immunoblotting analyses using anti-IκB antibody. The basal and phosphorylated forms of HA-IκBα are indicated as IκBα and IκBα-P, respectively. NS indicates a nonspecific band cross-reacted with the anti-HA antibody. C, immunoblotting analysis showing the expression level of transfected NIK and NIK mutants. The HA-tagged wild type (WT) and C-terminal truncated forms of NIK (0.5 μg) were transfected into 293 cells, and the expressed proteins were detected by immunoblotting using anti-HA. The multiple bands shown in lanes 2–4 represent the hyper- and hypophosphorylated NIK (data not shown). NS indicates a nonspecific band. D, luciferase reporter gene assays to determine the sequences of NIK required for activation of NF-κB transcriptional activation activity. Jurkat cells (1.25 × 10⁶) were transfected with the eB-TATA-luc reporter gene (50 ng) together with the wild type (WT) or the indicated truncated forms of NIK (50 ng). After 40 h, the transfectants were collected for extract preparation and luciferase assays. Luciferase activity is presented as -fold induction relative to the basal level measured in cells transfected with the empty vector pCMV4. The values are means ± standard errors from three independent experiments.

Luciferase substrate and then immediately measured with a single photon channel of a scintillation counter (Beckman).

IκBα-P). Deletion of 100 amino acids from the C terminus of NIK did not significantly inhibit its IKK activation function (Fig. 1B, lanes 5 and 6). However, further deletion of 52 amino acids or more from this end resulted in the abrogation of the signaling function of NIK (lanes 5–8 and 13–16). The failure of these NIK mutants to induce IkBα phosphorylation was not due to their inefficient expression or low stability since the steady expression level of these NIK mutants was similar to that of the wild type NIK (Fig. 1C).

The functional phenotypes of the NIK mutants were further assessed using the more quantitative reporter gene assays. As shown in Fig. 1D, expression of NIK in Jurkat T cells led to significant induction of the eB-directed luciferase reporter gene expression (column 2). Interestingly, deletion of the C-terminal 100 amino acids of NIK led to a partial inhibition of NIK function (column 0). No NF-κB inducing activity was detected with the other NIK mutants lacking more C-terminal sequences (columns 4–7). Thus, the C-terminal non-catalytic region of NIK is essential for its signaling function.

The N-terminal Sequences of NIK Exhibit Negative-regulatory Function—The N-terminal non-catalytic region of NIK contains 400 amino acids (19). To examine the role of this region in NIK function, N-terminal truncation mutants of NIK were subjected to functional assays (Fig. 2). In contrast to the C-terminal region, the majority of the N-terminal sequences appeared to be dispensable for NIK function (Fig. 2B). Deletion of up to 348 amino acids did not block the function of NIK in

Fig. 2. The N-terminal sequences of NIK are largely dispensable for its signaling function. 
A, schematic summary of the N-terminal truncation mutants of NIK. The catalytic domain is shown in black boxes. B, IkBα phosphorylation in 293 cells induced by IKKα and NIK. 293 cells were transfected with HA-tagged IkBα (0.1 μg), HA-tagged wild type (WT), or truncated forms of NIK (50 ng) together with IKKβ (10 ng). The basal and phosphorylated forms of IkBα were detected by immunoblotting with anti-IκB and indicated as IκBα and IκBα-P, respectively. The lower level of IkBα in lanes 6–8 appeared to result from higher levels of IkBα phosphorylation and degradation, since a significantly higher level of IkBα was detected when the cells were incubated with a proteasome inhibitor, MG132 (data not shown). NS indicates a nonspecific band cross-reacted with the anti-IκB antibody. C, immunoblotting analysis showing the expression level of transfected NIK and NIK mutants. The HA-tagged wild type (WT) and N-terminal truncated forms of NIK (0.5 μg) were transfected into 293 cells, and the expressed proteins were detected by immunoblotting using anti-HA. The multiple bands detected for each of these mutants resulted from constitutive phosphorylation at their C-terminal region (data not shown). NS indicates nonspecific bands. D, luciferase reporter gene assays. Jurkat T cells were transfected with eB-TATA-luc together with the indicated amounts of wild type (wt) or N-terminal truncated forms of NIK. Luciferase activity was determined and presented as described in Fig. 1D.
The PRR shown in \textit{C} forms. PRR motifs of NIK.

repeat (PRR) and proline-rich

mary of the kinase domain and the basic region (BR), as demonstrated by \textit{in vivo}

activation of IKK\(\beta\) (Fig. 2B, lanes 3–9) and IKK\(\alpha\) (data not shown), as demonstrated by \textit{in vivo} phosphorylation of IkBa.

Complete inactivation of NIK was observed only when 377 or more amino acids were deleted (lanes 10 and 11). Similar results were obtained with luciferase reporter gene assays (Fig. 2D). More interestingly, we observed that several of the N-terminal NIK truncation mutants (152–947, 238–947, and 319–947) exhibited significantly higher activity in activation of IKK\(\beta\) (Fig. 2B, lanes 6–8) and induction of \(\beta\) (Fig. 2D, columns 5–7) than the wild type form. This functional elevation was even more profound when lower amounts of expression vectors were used in the transfections (Fig. 2D, columns 12–15). Moreover, the differential activity of the NIK mutants was not due to the variation in their expression, since the more active forms of NIK were expressed at either equivalent or even lower levels compared with the wild type NIK (Fig. 2C). These results suggest that the N-terminal region of NIK functions as a negative-regulatory domain (NRD) and that the core sequences of this domain likely reside between amino acids 121 and 318.

The NRD of NIK Contains Two Negative-regulatory Motifs—Sequence analyses of the negative-regulatory region of NIK revealed two interesting structural motifs (Fig. 3A). The first motif is similar to the basic region (BR) of basic leucine zipper (bZIP) motifs present in various transcription factors, such as GCN4 and members of the Fos/Jun and CREB/ATF families (26–28). The bZIP motif is composed of a BR and a downstream leucine zipper. The NIK motif contains a perfect BR, although it lacks a leucine zipper (Fig. 3B). The second structural domain observed in the negative-regulatory region of NIK is a proline-rich repeat (PRR) sequence located between amino acid 250 and amino acid 317 (Fig. 3A). This domain is composed of a number of short repeats, which share a consensus sequence PXXFPX (Fig. 3C). Additionally, repeats 4 and 5 share many other identical amino acid residues (Fig. 3C, see sequence alignment).

To determine the role of these motifs in regulation of NIK function, internal deletions were performed to selectively remove each of these structural sequences (Fig. 4A). Luciferase reporter gene assays revealed that deletion of the BR led to a marked increase in the NF-\(\beta\)-inducing function of NIK (Fig. 4C, \(\Delta BR\)). Furthermore, removal of the PRR motif or PRR together with the BR resulted in even more striking increase in the NF-\(\beta\) inducing activity of NIK (\(\Delta PRR\) and \(\Delta BR/\Delta PRR\)). Parallel immunoblotting assays showed that the expression levels of the NIK deletion mutants were either similar or even lower than that of the wild type NIK (Fig. 4B). Thus, both the BR and the PRR motifs appear to play a negative-regulatory role in controlling the signaling function of NIK.

The N-terminal Fragment of NIK Inhibits NIK-mediated NF-\(\beta\) Signaling in Trans—We next examined whether expression of the N-terminal fragment of NIK is able to inhibit the NF-\(\beta\)-inducing activity of this kinase in trans. For these studies, a NIK N-terminal fragment (NIK-(1–366)) containing the negative-regulatory motifs was coexpressed with various “super-active” NIK molecules lacking the N-terminal NRD (NIK-(152–947), NIK-(238–947), and NIK-(319–947)), together with the \(\beta\)-TATA-luc reporter. In the absence of NIK-(1–366), all the super-active NIK proteins potently stimulated the \(\beta\)-specific luciferase expression (Fig. 5A). Remarkably, expression of NIK-(1–366) led to a dose-dependent inhibition of the \(\beta\) activation. The specificity of this inhibitory effect was demonstrated by the finding that NIK-(1–366) did not inhibit \(\beta\) activation mediated by the dominant-active MEKK1 (Fig. 5A, columns 15–17). To examine the role of the BR and PRR motifs in the negative regulation of NIK, N-terminal fragments of NIK lacking these motifs were subjected to the trans-inhibition assays. When the BR and PRR motifs were deleted separately, the resulted N-terminal fragments of NIK (NIK-(1–366)\(\Delta BR\) and NIK-(1–366)\(\Delta PRR\)) partially lost their inhibitory effect on NIK function (Fig. 5B, columns 2–7). Deletion of the PRR together with BR completely blocked the inhibitory action of the NIK NRD (Fig. 5B, columns 8–10). Immunoblotting assays revealed that the NIK-(1–366) also inhibited NIK-induced IkBa phosphorylation, and this inhibitory action required the BR and PRR motifs (Fig. 5C). Together, these results strongly

*Fig. 3. Summary of sequence motifs of NIK. A, schematic summary of the kinase domain and the basic region (BR) and proline-rich repeat (PRR) motifs of NIK. B and C, sequences of the individual motifs. The PRR shown in C is presented as both linear (sequence) and aligned (sequence alignment) forms. LZ, leucine zipper.*

*Fig. 4. Identification of N-terminal motifs of NIK exhibiting negative-regulatory functions. A, schematic picture of NIK mutants lacking the basic region (\(\Delta BR\)), proline-rich repeat (\(\Delta PRR\)), and both BR and PRR (\(\Delta BR/\Delta PRR\)). Potential structural domains are shown as black boxes. B, immunoblotting analysis showing the expression levels of transfected NIK and its internal deletion mutants. 293 cells were transfected with either the pCMV-\(\Delta HA\) empty vector (Vector) or the indicated HA-tagged NIK constructs (0.5 \(\mu\)g), and the expressed proteins were analyzed by immunoblot with anti-HA. WT, wild type; C, sequence motifs negatively affecting function of NIK in NF-\(\beta\)-activation. Jurkat T cells were transfected with \(\beta\)-TATA-luc together with the indicated wild type (\(wt\)) or internal deletion mutants of NIK (50 ng). Luciferase activity was determined and presented as in Fig. 1D.*
suggest that the signaling function of NIK is negatively regulated by its N-terminal structural motifs, including the BR and PRR.

The NRD Interacts with the C Terminus of NIK and Inhibits NIK/IKK Interaction—To investigate the mechanism by which the NRD inhibits NIK function, we examined whether the NRD of NIK interacts with other regions of this MAP3K. For these studies, NIK-(1–366) was expressed in 293 cells together with wild type NIK (NIKwt) or various NIK truncation mutants, followed by co-immunoprecipitation assays. As expected from a previous study (20), NIK-(1–366) physically associated with the NIKwt since these proteins were coprecipitated from a previous study (20), NIK-(1–366) physically associates with the NIKwt (upper panel). The expression level of the Myc-tagged NIK-(1–366) or derivatives was determined by immunoblotting assay using the anti-Myc antibody (lower panel).

Fig. 5. Inhibition of NIK function by its N-terminal fragment. A, Jurkat T cells were transfected with the indicated NIK constructs or dominant-active MEKK1 (0.1 μg) together with increasing amounts of NIK-(1–366). The cells were also transfected with the eB-TATA-Luc reporter plasmid. Luciferase activity is presented as -fold induction relative to the basal level measured in cells transfected with an empty vector (column 1). The data are representative of three independent experiments. B, Jurkat T cells were transfected with the eB-TATA-Luc reporter and NIK-(152–947) together with increasing amounts of NIK-(1–366) lacking the BR, PRR, or both BR and PRR. Luciferase activity was determined and presented as in A. C, inhibition of NIK-mediated IKKα activation by the NRD. 293 cells were transfected with the indicated expression vectors together with HA-tagged IκBα as described in Fig. 1. Phosphorylation of IκBα was detected by immunoblotting with anti-HA antibody (upper panel). The basal and phosphorylated forms of HA-IκBα are indicated as IκBα and IκBα-P, respectively. The expression level of the Myc-tagged NIK-(1–366) or derivatives was determined by an immunoblotting assay using the anti-Myc antibody (lower panel).

The specificity of this molecular interaction was demonstrated by the lack of NIKwt precipitation in the absence of NIK-(1–366) (lane 1). More importantly, deletion of up to 650 amino acids from the N terminus of NIK did not affect its interaction with the NIK-(1–366) (lanes 3–5). On the other hand, removal of 100 or more amino acids from the C terminus of NIK completely abolished the association of this kinase with its N-terminal region (lanes 6 and 7). Parallel immunoblotting assays revealed that the different NIK mutants were expressed at similar levels (Fig. 6, lower panel). Thus, the N-terminal NRD specifically interacts with the C-terminal region of NIK.

Since the C terminus of NIK is involved in binding to IKK (20), the finding described above suggested the possibility that the N-terminal NRD of NIK may interfere with the NIK/IKK interaction. To examine this possibility, NIK-(152–947) was cotransfected with IKKα into 293 cells along with an increasing amount of the N-terminal fragment of NIK (NIK-(1–366)). In the absence of NIK-(1–366), NIK-(152–947) was readily copre-
Negative Regulation of NIK

Activation of NF-κB by various cellular stimuli is triggered by IKK-mediated phosphorylation of IkBα. Recent studies suggest several potential IKK activating kinases, including NIK and MEKK1 (16–18). Both MEKK1 and NIK belong to the MAP3K family, which share extensive sequence homology in their kinase domains. It is believed that MAP3Ks receive upstream signals and then phosphorylate and activate downstream kinases, the mitogen-activated protein kinase kinases (29). MEKK1 is composed of a C-terminal catalytic domain and a large N-terminal regulatory domain (30). Activation of this MAP3K is mediated through its phosphorylation by upstream kinases (31) or by caspase-mediated proteolytic cleavage involving the removal of its N-terminal regulatory domain (32). The mechanism by which NIK is regulated has remained unknown. In the present study, we show that NIK also contains an NRD, although this region is shorter than the regulatory domain of MEKK1. The NIK NRD appears to function by interacting with the C-terminal region of this MAP3K. When expressed as an N-terminal fragment, the NRD forms a stable complex with the C-terminal fragment of NIK (Fig. 6). It is possible that this intramolecular interaction may induce conformational changes in NIK, which affects the function of its kinase domain. It is also likely that the N-terminal NRD interferes with the binding of NIK to its substrate IKK, since the IKK binding is mediated by the C terminus of NIK. Indeed, when overexpressed, the N-terminal fragment of NIK strongly inhibits the NIK/IKK interaction (Fig. 7). The NIK NRD contains two visible sequence motifs, BR and PRR, which contribute to the negative-regulatory function of this cis-acting domain. The BR shares remarkable sequence similarities with the basic region of the GCN-4 bZIP (see Fig. 3B). Both the BR and PRR are important for the physical interaction of the NRD with the C terminus of NIK. Interestingly, we have observed that the C-terminal region of NIK undergoes phosphorylation at multiple sites, generating protein bands with different mobility (Fig. 6, lane 5, and data not shown). It remains to be examined whether the phosphorylated residues participate in binding with the basic residues in the BR located in the NRD. Although the precise mechanisms mediating the function of the BR and PRR motifs require further studies, the data presented in the current study demonstrate that NIK is regulated by a cis-acting domain.

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Additions and Corrections

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The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform.

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Page 14909: Line 14 of the Abstract should read “with high affinity (K_a approximately 7 nM) . . . .”

Page 14914, line 3 and in the legend to Fig. 5: “EC_50 of 7.4 ± 1.1 pM” should be “EC_50 of 7.4 ± 1.1 nM.” Also, the x axis in Fig. 5B should be revised so that the values are increased 1000-fold (i.e. the range becomes 10^(-9) to 10^(-5) instead of 10^(-12) to 10^(-8)). The revised Fig. 5 and its legend are shown below.

These corrections do not affect the conclusions of the paper.

Fig. 5. Interaction of recombinant forms of PDE4D5 and RACK1, as purified from E. coli. A, fusions between GST and RACK1, and also between MBP and PDE4D3 or PDE4D5, were expressed and purified from E. coli (see “Experimental Procedures”). GST alone (i.e. not as a fusion) was expressed and purified in an identical manner. Cell lysates (lanes b) and purified proteins (lanes c) obtained after elution from the appropriate affinity column were run on SDS-PAGE and stained with Coomassie Blue. The species were purified to apparent homogeneity as analyzed by SDS-PAGE. The positions of the arrows mark the relative molecular weight of the purified proteins as follows: GST, 27.3 ± 1.1 kDa; GST-RACK1, 59.8 ± 1.4 kDa; MBP-4D3, 128 ± 3.8 kDa; MBP-4D5, 155.3 ± 2.6 kDa. These data are typical of experiments done at least three times. B, the interaction of E. coli-purified recombinant PDE4D5 and RACK1 was tested in an ELISA as described under “Experimental Procedures.” The MBP-PDE4D5 fusion bound to GST-RACK1 in a dose-dependent manner, with an EC_50 of 7.4 ± 1.1 nM (mean ± S.D.; n = 3 separate experiments). As a control, parallel experiments were performed for MBP-PDE4D3 (“4D3-MBP”) alone, and also on MBP-PDE4D5 complexed with GST-RACK1. Assays were performed using an excess of GST-RACK1 so that all of the PDE4D5 would be complexed with RACK1 (see “Experimental Procedures”). In pull-down experiments, all of the PDE4D5 could be shown to complex with RACK1 under these conditions (data not shown). As a control, assays were performed with GST alone, added at comparable levels. The IC_50 values for rolipram inhibition were 0.13 ± 0.05, 0.16 ± 0.05, and 0.52 ± 0.07 μM for MBP-PDE4D5 alone, MBP-PDE4D5 mixed with GST, and MBP-PDE4D5 mixed with GST-RACK1, respectively (mean ± S.D., n = 3). These values are significantly different (MBP-PDE4D5 alone compared with MBP-PDE4D5 complexed with GST-RACK1; p < 0.005, t test).

Protein assays were performed, and molar concentrations were determined on the basis of the calculated molecular weights.

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FIG. 1. Determination of C-terminal sequences of NIK required for its function in the induction of IkBα phosphorylation. A, schematic summary of C-terminal truncation mutants of NIK. The catalytic domain is shown in black boxes. B, IkBα phosphorylation in 293 cells induced by IKKs and NIK. 293 cells were transfected (in 24-well plates) with HA-tagged IkBα (0.1 μg), HA-tagged wild type (WT) or mutant forms of NIK (50 ng), together with either IKKa (25 ng, lanes 2–8) or IKKβ (10 ng, lanes 10–16). Whole cell extracts were subjected to immunoblotting analyses using anti-HA antibody. The basal and phosphorylated forms of HA-IkBα are indicated as IkBα and IkBα-P, respectively. NS indicates a nonspecific band cross-reacted with the anti-HA antibody. C, immunoblotting analysis showing the expression level of transfected NIK and NIK mutants. The HA-tagged wild type (WT) and C-terminal truncated forms of NIK (0.5 μg) were transfected into 293 cells, and the expressed proteins were detected by immunoblotting using anti-HA. The multiple bands shown in lanes 2–4 represent the hyper- and hypophosphorylated NIK (data not shown). NS indicates a nonspecific band. D, luciferase reporter gene assays to determine the sequences of NIK required for activation of NF-κB transcriptional activation activity. Jurkat cells (1.25 × 10⁶) were transfected with the κB-TATA-luc reporter gene (50 ng) together with the wild type (WT) or the indicated truncated forms of NIK (50 ng). After 40 h, the transfectants were collected for extract preparation and luciferase assays. Luciferase activity is presented as -fold induction relative to the basal level measured in cells transfected with the empty vector pCMV4. The values are means ± standard errors from three independent experiments.