Mol. Cells, Vol. 18, No. 2, pp. 200-206

Molecules and Cells

IKKγ Inhibits Activation of NF-κB by NIK

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(Received June 3, 2004; Accepted July 9, 2004)

IKKγ is a component of the IKK complex, which regulates NF-κB activity. To investigate the role of IKKγ, we expressed wild type IKKγ containing 412 amino acids, and deletion mutants containing residues 1-312 and 101-412, using murine IKKγ cDNA. In a co-transfection assay with a CAT reporter plasmid, NIK activated NF-κB-dependent gene expression approximately two fold and this expression was inhibited by co-transfection of a wild type IKKγ expression plasmid. In binding assays IKKγ inhibited the association of IκBα with IKKβ and the subsequent phosphorylation of IκBα that is activated by NIK. Inhibition by IKKγ also occurred in an assay with a dominant negative mutant of NIK but not with a C-terminal deletion mutant of IKKγ, indicating that the C-terminal 100 amino acids of IKKγ are important for negative regulation of NF-κB activation. In addition, the interaction of IKKβ with IKKγ was inhibited by co-transfection with a NIK expression plasmid. Our results suggest that overexpression of IKKγ inhibits activation of NF-κB by NIK by competing with NIK for interaction with IKKβ.

Keywords: IκB Kinases; IKK Complex; IKKγ; NF-κB Activation; NIK; Signal Transduction.

Introduction

The transcription factor NF-κB regulates numerous genes involved in cellular signaling, cell growth, survival and apoptosis (Baldwin, 1996; Barnes, 1997; Ghosh *et al*., 1998). It is activated by proinflammatory stimuli such as bacterial and viral infection, cytokines, and double-stranded RNA, and upregulates genes involved in inflammation (Hoffmann *et al*., 2002; Karin, 1999; Kim *et al*., 2004; Li and Gaynor, 1999). Constitutive activation of NF-κB has been described in many types of solid tumors including breast cancer, melanoma, colon cancer and pancreatic cancer, and this activation appears to affect cancer cell survival (Dejardin *et al*., 1999; Devalaraja *et al*., 1999; Schwartz *et al*., 1999; Wang *et al*., 1999). The rapid activation and subsequent silencing of the NF-κB pathway in response to a variety of extracellular stimuli suggests that it is controlled both positively and negatively. The potential of NF-κB as a drug target in inflammatory diseases and cancer underlines the importance of further characterization of the mechanisms that regulate the NF-κB pathway.

NF-κB is normally sequestered in the cytoplasm where it is bound by a family of inhibitory proteins known as IκB (Beg *et al*., 1992; Henkel *et al*., 1992). A variety of stimuli which activate NF-κB increase the activity of two related kinases, IKKα and IKKβ, and result in the phosphorylation and subsequent degradation of IκB proteins by the proteasome (Chen *et al*., 1995; DiDonato *et al*., 1996; Traenckner *et al*., 1995). This leads to nuclear

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Abbreviations: CMV, cytomegalovirus; HA, hemagglutinin; IκB, inhibitor of NF-κB; IKK, IκB kinase; NF-κB, nuclear factor-κB; NIK, NF-κB inducing kinase.

translocation of NF-κB, which then binds to consensus sequences upstream of target genes (Beg *et al*., 1995; Stancovski and Balimore, 1997). IKKα and IKKβ are components of the high molecular weight IKK complex, together with IKKγ (Zandi *et al*., 1997). Extracellular stimuli cause upstream kinases including the mitogen-activated protein kinase kinase family members, NIK and MEKK1, to activate IKKα and IKKβ by phosphorylating serine residues in their activation loops (Ling *et al*., 1998; Yin *et al*., 1998a).

IKKγ (also named NF-κB essential modulator, NEMO) is critical component of the IKK complex required for proinflammatory activation of NF-κB (May *et al*., 2000). This 48 kDa glutamine-rich protein contains a leucine zipper domain and two coiled-coil motifs, and binds IKKβ through its amino-terminal domain (Royet *et al*., 2000; Zang *et al*., 2000). The interaction of IKKγ with IKKα and IKKβ is critical for the formation of the high molecular weight IKK complex and for activation of IKKβ by proinflammatory stimuli (Li *et al*., 2001; Yamamoto *et al*., 2001). Although IKKγ itself does not have kinase activity, it is essential for NF-κB activation (Yamaoka *et al*., 1998). Disruption of the single copy of IKKγ, on the X chromosome, resulted in the death of male mice due to hepatic apoptosis, while female mice developed granulocytic infiltration and increased apoptosis of keratinocytes (Makris *et al*., 2000). Human subjects with a mutation of the single copy of IKKγ develop a severe skin disease known as *incontinentia pigmentia* (Rudolph *et al*., 2000; Schmidt-Supprian *et al*., 2000). These observations indicate that IKKγ plays a central role in regulation of the NF-κB pathway. But the detailed mechanism by which it regulates NF-κB activity is not clear.

In this study, we expressed the wild type and deletion mutants of IKKγ and investigated their effects on the activation of NF-κB.

Materials and Methods

Cell culture and transfection COS cells, an African green monkey kidney cell line transformed by SV40, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and maintained in 5% CO_2 at 37°C. Subconfluent cultures of the cells were transfected with plasmid DNA (10–20 μg/10 cm plate) using Fugene 6 (Roche Molecular Biochemicals). The plasmid DNA was mixed with Fugene 6 in serum-free DMEM for 15 min at room temperature and applied to culture plates as described by the manufacturer.

CAT assay The chloramphenicol acetyltransferase (CAT) assay was performed according to Gorman *et al*. (1982). After 48 h of transfection with the CAT plasmid, the cells were rinsed twice with PBS and harvested by scraping with a rubber policeman. The cells suspended in Tris-HCl (pH 7.8) were disrupted by five cycles of freeze-thawing. After removal of cell debris by centrifugation for 15 min at 4°C in a microcentrifuge, the supernatant was used as a cell extract and assayed for protein content by the Bradford method (Bradford, 1976). An aliquot of the extract was incubated with 0.2 μCi[^{14}C]-chloramphenicol (Amersham) and 1 mM acetyl CoA (Sigma) in 0.25 M Tris-HCl (pH 7.8) at 37°C. The reaction products were extracted with ethyl acetate and separated by ascending thin-layer chromatography using chloroform:methanol (95:5). Radioactivity was localized on X-ray films by autoradiography and subjected to density analysis using an Image Analysis System (Bio-Rad).

Preparation of cytoplasmic extracts Cytoplasmic extracts were prepared according to Dignam *et al*. (1983) with slight modifications. Cells, washed twice with cold PBS, were harvested by scraping from culture dishes and pelleted by centrifugation at 1,500 rpm for 10 min. The pellets were resuspended in buffer A [10 mM Hepes (pH 7.9), 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol] supplemented with proteinase inhibitors (Roche Molecular Biochemicals). After incubation on ice for 15 min, they were disrupted by 20 passages through a 25-gauge needle and the resulting suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was assayed for protein and used for immunoprecipitation and Western blotting.

Immunoprecipitation and Western blotting Cytoplasmic extracts of the COS cells were incubated with 2.5 μg of monoclonal antibody against the HA epitope (Santacruz Biotechnology) for 2 h at 4°C. After addition of 20 μl of protein A agarose beads (Bio-Rad) and incubation for 1 h at 4°C, the immunoprecipitates were washed three times with modified buffer D [20 mM Hepes (pH 7.9), 100 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.06% Nonidet-P-40] containing proteinase inhibitors. They were then run on a 10% SDS-polyacrylamide gel and the gel was Western blotted with specific antibodies and chemiluminescence reagents (Amersham Pharmacia Biotech.). Monoclonal antibodies used were against the Flag epitope (Sigma), Myc epitope (Santa Cruz Biotechnology) and HA epitope (Santa Cruz Biotechnology).

Electrophoretic mobility shift assay (EMSA) COS cells transfected with the indicated expression plasmids were harvested and nuclear extracts prepared as described previously (Kim *et al*., 2000). Equal amounts of the nuclear proteins were incubated with the [^{32}P]-labeled double stranded oligonucleotide, 5′-GGGCCAAGAATCTTAGCAGTTTCGGG-3′, for 30 min. The reaction products were electrophoretically separated on 4.5% native polyacrylamides gels in TBE buffer [44.5 mM Tris (pH 8.0), 44.5 mM boric acid and 1 mM EDTA] for 3 h at 120 V, and the gels dried and autoradiographed.

Results

Expression of wild type and deletion mutants of IKKγ

IKKγ contains several distinct domains including an amino-terminal domain which mediates its interaction with IKKβ, a coiled-coil domain which is important for oligomerization, a leucine zipper of as yet uncharacterized function and the carboxy-terminus which recruits upstream kinases (Li *et al*., 2001). In order to investigate its regulatory function in the activation of NF-κB, we constructed expression plasmids of carboxy-terminal (⊿C) and amino-terminal (⊿N) deletion mutants (Fig. 1A). Wild type murine IKKγ cDNA was cloned into the CMV expression vector, pCMV5, fusing the Myc tag to the amino terminus of the IKKγ sequence (Yamamoto *et al*., 2001). The C-terminal deletion containing amino acid residues 1-312, and the N-terminal deletion containing residues 101–412 of IKKγ, were also cloned downstream of the Myc epitope in pCMV5 (Fig. 1A). The resulting plasmids were transfected into COS cells and expressed IKKγ proteins detected by Western blotting (Fig. 1B). Using monoclonal antibodies against the C-terminus and N-terminus of IKKγ, the deletion of each mutant was confirmed (Fig. 1B).

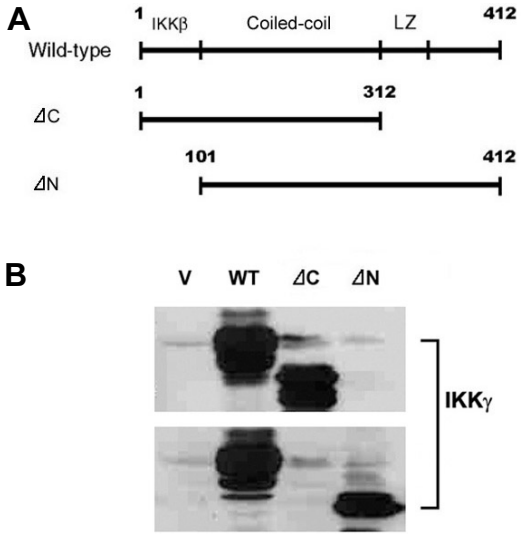

Fig. 1. Schematic illustration of the domains included in the expression plasmids for the wild-type, ⊿C and ⊿N IKKγ proteins. **A.** The N-terminal IKKβ binding domain, coiled-coil domain and leucine zipper domain (LZ) are shown. **B.** Cytoplasmic extracts of COS cells transfected with pCMV5 empty vector (V) or each IKKγ expression plasmid were electrophoresed and analyzed by Western blotting using monoclonal antibodies against the N-terminal (upper) or C-terminal (lower) region of IKKγ.

Overexpression of IKKγ inhibits activation of NF-κB by NIK The activation of NF-κB by extracellular stimuli is triggered by activation of the IKK complex; an increase in the activity of the IKK complex leads to phosphorylation and subsequent degradation of IκB and activation of the NF-κB pathway (Chen *et al*., 1995; DiDonato *et al*., 1996). Two members of the MAP3K family, NIK and MEKK1, have been identified (Ling *et al*., 1998) within the high molecular weight IKK complex that migrates between 600 and 900 kDa (Yamamoto *et al*., 2001). These kinases phosphorylate the serine residues in the activation loop of IKKβ and stimulate its kinase activity (Yin *et al*., 1998a). Several lines of evidence suggest that the phosphorylation of IKKβ by NIK or MEKK1, together with autophosphorylation by IKKβ itself, is the critical event that leads to activation of NF-κB in response to proinflammatory stimuli (Traenckner *et al*., 1995). Thus, IKK activity is induced in cells overexpressing either NIK or MEKK1 (Malinin *et al*., 1997). In addition, NIK associates with IKKβ and IKKγ, and IKKγ is known to be essential for activation of NF-κB (Rothwarf *et al*., 1998). However the detailed function of IKKγ in activation of NF-κB is not clear. To determine the role of IKKγ, we first investigated its effect on the activation of NF-κB by NIK. Using a CAT reporter plasmid driven by the tk-promoter of herpes simplex virus combined with four NF-κB binding domains, we estimated NF-κB activation via the CAT assay. Expression of *CAT* was increased about 2-fold by transfection with an expression plasmid for wild type NIK (Fig. 2), and this activation was inhibited by approximately 50% by co-transfection with an expression plasmid for wild type IKKγ (Fig. 2). But not with an expression plasmid for the C-terminal IKKγ deletion mutant (⊿C) (Fig. 2). The N-terminal IKKγ deletion mutant (⊿N) had a slightly lower effect on activity than wild type IKKγ (Fig. 2). We also used a dominant negative mutant of NIK in this assay that lacks kinase activity and suppresses endogenous NIK function (Yin *et al*., 1998b). *CAT* expression was decreased by about 50% by co-transfection with the expression plasmid for this mutant (Fig. 2) and the two deletion mutants of IKKγ had parallel effects to those that they had with wild type NIK (Fig. 2). These results indicate that IKKγ has a negative regulatory role in the activation of NF-κB by NIK and its C-terminal 100 amino acids are important for this effect.

Association of IκBα with NIK-activated IKKβ is inhibited by IKKγ In order to further explore the role of IKKγ in the activation of NF-κB by NIK, expression plasmids encoding HA-tagged IKKβ, Flag-tagged IκBα, Myc-tagged IKKγ and Myc-tagged NIK were transfected in various combinations into COS cells and the interaction of IκBα with IKKβ was assayed. Following immunoprecipitation of HA-IKKβ with cytoplasmic extracts of the transfected cells, we performed Western blot analysis using epitope-specific antibodies directed against IκBα, IKKγ or NIK (Fig. 3A). When HA-IKKβ and Flag-IκBα were co-transfected, a small amount of Flag-IκBα was associated with the immunoprecipitated HA-IKKβ (Fig. 3A, lane 2, top panel). As expected, co-transfection

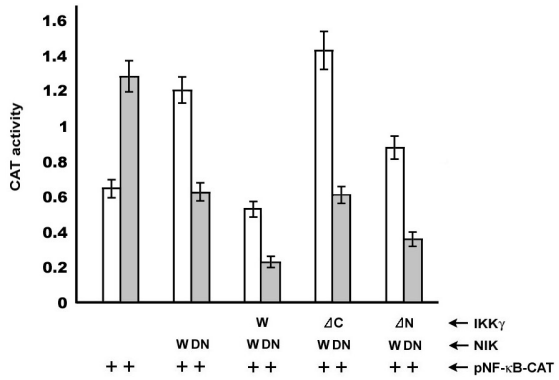

Fig. 2. Effect of IKKγ on the activation of NF-κB by NIK. The CAT reporter plasmid (pNF-κB-CAT) was co-transfected into COS cells with the wild type expression plasmid (W, white boxes) or the dominant negative (DN, dark boxes) mutant of NIK, and the expression plasmid for the wild type (W) or deletion mutant (⊿C or ⊿N) of IKKγ, as indicated. 48 h post-transfection, the cells were harvested and assayed for CAT.

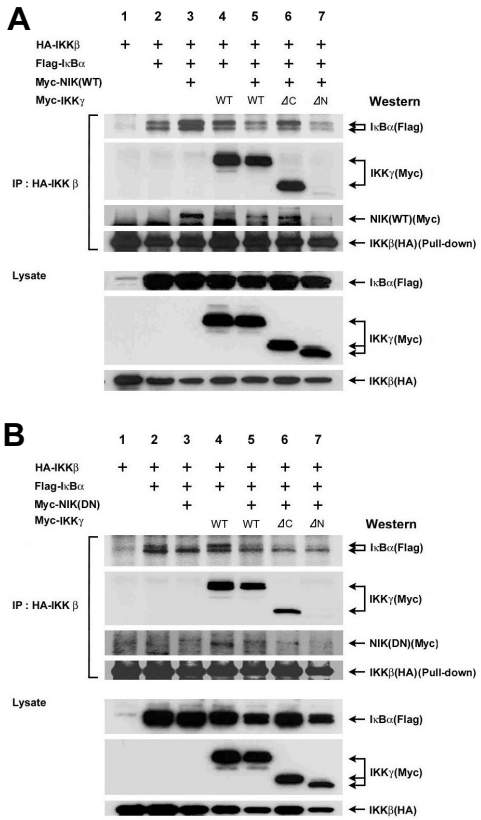

Fig. 3. Effect of IKKγ on the association of IκBα with IKKβ in the presence of wild type (A) or the dominant negative mutant (B) of NIK. Expression plasmids of HA-tagged IKKβ, Flag-tagged IκBα, Myc-tagged wild type (WT) NIK (in A) or the Myc-tagged dominant negative mutant (DN) of NIK (in B) and Myc-tagged wild type (WT) or deletion mutants (⊿C, ⊿N) of IKKγ were transfected into COS cells in various combinations as indicated. After 48 h, cytoplasmic extracts were prepared and used for immunoprecipitation with anti-HA monoclonal antibody (top three panels in A and B), and the associated proteins were assayed by Western blotting. The expression level of each protein in the lysates before immunoprecipitation was determined by Western blotting (lower three panels in A and B).

of Myc-NIK, HA-IKKβ, and Flag-IκBα resulted in increased association of Flag-IκBα with the immunoprecipitated HA-IKKβ (Fig. 3A, lane 3, top panel). The amount of phosphorylated Flag-IκBα was also increased by transfection of Myc-NIK, indicating that NIK stimulates IKKβ activity (Fig. 3A, upper band, lane 3, top panel). Wild type IKKγ also promoted the association of Flag-IκBα with HA-IKKβ, although the effect was lower than with NIK (Fig. 3A, lane 4, top panel). But when Myc-NIK was co-transfected with wild type Myc-IKKγ, HA-IKKβ, and Flag- IκBα, the stimulatory effect of NIK on the association of IκBα with IKKβ did not occur. This result again indicates that IKKγ inhibits activation of IKKβ by NIK (Fig. 3A, lane 5, top panel). Inhibition was also evident when the N-terminal deletion mutant (⊿N) of IKKγ was co-transfected (Fig. 3A, lane 7, top panel), but not with the C-terminal deletion (Fig. 3A, lane 6, top panel). Both wild type IKKγ, and the C-terminal deletion (⊿C), but not the N-terminal deletion, co-immunoprecipitated with IKKβ, indicating that the N-terminal region of IKKγ, as reported previously (Yamamoto *et al*., 2001), is necessary for the formation of a complex of IKKβ, IKKγ, and IκBα (Fig. 3A, lanes 4–7, second panel). Furthermore, the degree of inhibition of the interaction between IKKβ and NIK by wild type IKKγ and the N-terminal deletion correlated with the extent of the interaction of IKKβ with IκBα (Fig. 3A, lane 3, lanes 5–7, third panel). These results indicate that IKKγ facilitates the interaction of the IKK complex with IκBα in unstimulated cells but acts as an inhibitor of the activation of NF-κB when it is triggered by NIK. Our results also suggest that IKKγ inhibits the interaction of IKKβ with NIK and the subsequent activation of IKKβ so that the association of IKKβ with IκBα is decreased.

To further characterize the role of IKKγ in the interaction of IκBα and IKKβ in the presence of NIK, we performed similar transfection assays with the dominant negative mutant of NIK used in Fig. 2. Flag-IκBα was able to associate with HA-IKKβ alone (Fig. 3B, lane 2, top panel), and this association was increased by expression of wild type Myc-IKKγ (Fig. 3B, lane 4, top panel). However, expression of the dominant negative mutant suppressed the phosphorylation of IκBα and its association with IKKβ (Fig. 3B, lane 3, top panel). In addition, co-transfection of wild type IKKγ and its C-terminal (⊿C) and N-terminal (⊿N) deletion mutants resulted in further inhibition of IKKβ activity (Fig. 3B, lanes 5–7, top panel). The association of IKKγ with IKKβ thus ap-

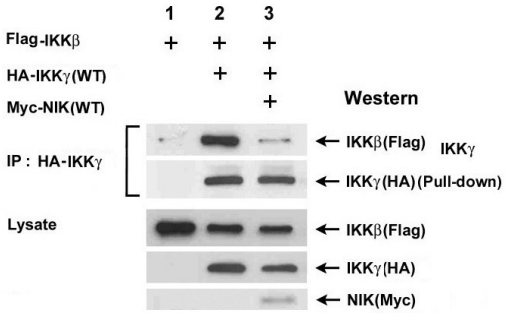

Fig. 4. Interaction of IKKγ with IKKβ in the presence of wild type NIK. The expression plasmid for Flag-tagged IKKβ was co-transfected with the expression plasmid for Myc-tagged wild type (WT) NIK or HA-tagged wild type (WT) IKKγ into COS cells as indicated. After 48 h, cytoplasmic extracts were prepared and immunoprecipitated with anti-HA monoclonal antibody, and the amounts of co-immunoprecipitated proteins were determined by Western blotting (top two panels). The expression level of each protein in the lysates before immunoprecipitation was determined by Western blotting (bottom three panels).

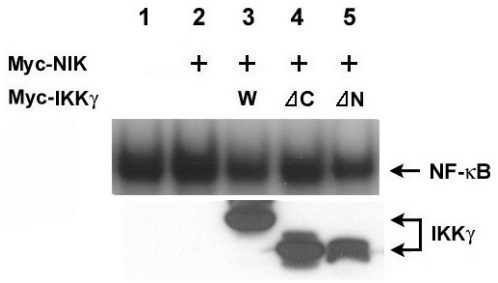

Fig. 5. IKKγ inhibits the nuclear translocation of NF-κB activated by NIK. The expression plasmid for Myc-tagged NIK was co-transfected into COS cells with the expression plasmid for Myc-tagged wild type (WT) or mutated IKKγ (⊿C or ⊿N). After 48 h, the cells were harvested and nuclear extracts prepared. EMSA was performed using [^{32}P]-labeled NF-κB consensus oligonucleotide (upper panel), and IKKγ in each nuclear extract was detected by Western blotting using anti-Myc monoclonal antibody (lower panel).

peared similar to that in the assay with wild type NIK (Fig. 3B, second panel). In contrast, the association of IKKβ with the dominant negative NIK was extremely weak compared to that with wild type NIK, indicating that the kinase activity of NIK is responsible for promoting this association (Fig. 3A, lane 3, third panel and Fig. 3B, lane 3, third panel). These results show that IKKγ inhibits NF-κB activation triggered by NIK and that this inhibition does not depend on the kinase activity of NIK.

NIK modulates the interaction of IKKγ and IKKβ We next examined whether NIK modulates the interaction between IKKβ and IKKγ. The expression plasmid encoding Flag-IKKβ was transfected alone or co-transfected with expression plasmids encoding wild type HA-IKKγ or wild type Myc-NIK into COS cells (Fig. 4). Cytoplasmic extracts were prepared and used for immunoprecipitation with monoclonal antibody against the HA epitope of IKKγ. When Flag-IKKβ and HA-IKKγ were co-transfected, there was considerable association of HA-IKKγ with Flag-IKKβ (Fig. 4, lane 2, top panel) but this was dramatically decreased by addition of Myc-NIK (Fig. 4, lane 3, top panel).

These results and the results showed in Fig. 3 (Fig. 3A, third panel) indicate that the association of IKKβ with NIK is inhibited by IKKγ, and that the association of IKKβ with IKKγ is inhibited by NIK.

Discussion

In this study, we explored the role of IKKγ in the activation of NF-κB by NIK. As reported by Yamamoto *et al.* (2001), we found that IKKγ activates IKKβ, facilitating the interaction of IKKβ with IκBα that results in increased phosphorylation of IκBα in cells in which NIK is not activated. Association of IKKβ with IκBα, and phosphorylation of IκBα, were also increased by transfection with a NIK expression plasmid, and led to activation of NF-κB. The phosphorylation of IKKβ by NIK is crucial for this activation (DiDonato *et al*., 1996; Traenckner *et al*., 1995). In the high molecular weight IKK complex of stimulated cells, NIK and IKKγ are found together with IKKs, and IKKγ is essential for formation of this complex (Yamamoto *et al*., 2001). These facts suggest that the roles of NIK and IKKγ in regulating IKK activity may be closely related. Several previous studies also indicate that IKKγ is essential for activation of NF-κB in response to extracellular stimuli (Royet *et al*., 2000; Yamaoka *et al*., 1998). Interestingly, we found here that activation of IKKβ and NF-κB by NIK is inhibited by overexpression of IKKγ. This suggests that levels of IKKγ that exceed those required for formation of the IKK complex and for NF-κB activation may actually inhibit NF-κB activation. As shown in Fig. 4, overexpression of IKKγ may trigger competition between IKKγ and NIK for association with IKKβ. In addition, we found that the C-terminal deletion mutant of IKKγ had no inhibitory effect on the activation of NF-κB although it is incorporated into the IKK complex. It is known that the C-terminal region of IKKγ is required for full formation and activation of the IKK complex in response to extracellular stimuli (Li *et al*., 2001; Rothwarf *et al*., 1998; Yamamoto *et al*., 2001). In contrast, the N-terminal deletion mutant retained the inhibitory effect on activation of NF-κB by NIK although it was unable to bind to IKKβ. These results indicate that inhibition of NF-κB activation may be due to the IKKγ which is not bound to IKKβ. However, the details of the

interactions between IKKγ, NIK, and IKKβ and the relationship between the formation of IKK complex and NF-κB regulation remain to be clarified.

Finally, we also confirmed inhibition of NF-κB activation by IKKγ in EMSAs with nuclear extracts of transfected cells. The binding of NF-κB to NF-κB consensus sequences was increased by expression of NIK (Fig. 5, lane 2, upper panel). But DNA binding was decreased by transfection of the plasmid expressing the wild type or N-terminal deletion mutant (⊿N) of IKKγ (Fig. 5, lanes 3 and 5, upper panel). Next, we examined the localization of IKKγ in the nucleus. Although the majority of IKKγ is present in the cytoplasm, we found that a small portion of IKKγ is present in the nucleus (Fig. 5, lower panel). It is not clear how the localization of IKKγ is controlled and further studies will be needed to address the possible function of IKKγ in the nucleus.

Acknowledgments This work was supported by grant R01-2001-000-00166-0 from the Basic Research Program of the Korea Science and Engineering Foundation.

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