IKKγ/NEMO Facilitates the Recruitment of the IκB Proteins into the IκB Kinase Complex

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IKKγ/NEMO is an essential regulatory component of the IκB kinase complex that is required for NF-κB activation in response to various stimuli including tumor necrosis factor-α and interleukin-1β. To investigate the mechanism by which IKKγ/NEMO regulates the IKK complex, we examined the ability of IKKγ/NEMO to recruit the IκB proteins into this complex. IKKγ/NEMO binding to wild-type, but not to a kinase-deficient IKKβ protein, facilitated the association of IκBo and IκBβ with the high molecular weight IKK complex. Following tumor necrosis factor-α treatment of HeLa cells, the majority of the phosphorylated form of endogenous IκBα was associated with the high molecular weight IKK complex in HeLa cells and parental mouse embryonic fibroblasts but not in IKKγ/NEMO-deficient cells. Finally, we demonstrate that IKKγ/NEMO facilitates the association of the IκB proteins and IKKβ and leads to increases in IκB kinase activity. These results suggest that an important function of IKKγ/NEMO is to facilitate the association of both IKKβ and IκB in the high molecular weight IKK complex to increase IκB phosphorylation.

The NF-κB proteins are critical for activating the expression of cellular genes that are involved in the control of the immune and inflammatory response and in protecting cells from apoptosis in response to a variety of stress stimuli (1–4). NF-κB is sequestered in the cytoplasm in most cells, where it is bound to a family of inhibitory proteins known as IκB (2, 5, 6). A variety of stimuli including the cytokines TNFα and interleukin-1, double-stranded RNA, and the viral transactivator Tax activate the NF-κB pathway (4, 7–11). These stimuli increase the activity of two related kinases, IKKα and IKKβ, to result in the phosphorylation of the IκB proteins (9, 12–16). A variety of studies using IKKα and IKKβ knock-out mice indicate that IKKβ is critical for NF-κB activation in response to cytokine treatment, whereas IKKα is not required for this function (17–22). The IκBo protein is phosphorylated on serine residues 32 and 36, while IκBβ is phosphorylated on serine residues 19 and 23, and this leads to their ubiquitination and degradation by the proteasome (10, 23–32). IκB mutants in which these serine residues are changed to alanine are resistant to proteasome-mediated degradation and thus prevent the nuclear translocation of the NF-κB proteins (33).

IKKγ/NEMO was initially identified in a genetic complementation assay as a factor that could restore NF-κB activation in cells that were resistant to a variety of stimuli that normally induce the NF-κB pathway (34). IKKγ/NEMO was also identified independently in biochemical studies as an essential component of the high molecular weight IKK complex (35, 36). Finally, this factor was characterized as a factor known as FIP-3 that bound to the adenovirus E3 protein and could inhibit TNFα–induced apoptosis (37). IKKγ/NEMO in conjunction with IKKα and IKKβ is a component of the high molecular weight IKK complex, which migrates between 600 and 900 kDa following gel filtration chromatography (12, 14, 29, 34–41). Biochemical fractionation and immunoprecipitation studies demonstrate that IKKα, IKKβ, and IKKγ/NEMO interact in this IKK complex (35, 36, 40). Cells that do not express IKKγ/NEMO are unable to assemble the high molecular weight IKK complex and increase IKK activity in response to agents that stimulate the NF-κB pathway (35, 36). Although IKKγ/NEMO itself does not have kinase activity, it is essential for NF-κB activation (34–36). The mechanism by which IKKγ/NEMO activates the NF-κB pathway has been the subject of intense investigation.

Mutagenesis of IKKγ/NEMO has been performed in an attempt to define important functional domains (35, 36, 42, 43). IKKγ/NEMO has a molecular mass of 48 kDa and contains a leucine zipper and two coiled-coil motifs. Residues in the amino-terminal 100 amino acids of this protein are critical for interactions with IKKβ (43). IKKβ preferentially associates with IKKγ/NEMO (34, 36), although IKKα has also been shown to directly associate with IKKγ/NEMO (22, 40). The coiled-coil domains in IKKγ/NEMO mediate its oligomerization, which is critical for activating IKK kinase activity (44), while its carboxyl terminus is involved in the recruitment of upstream kinases, which are critical for activating IKK (45). For example, a kinase known as RIP, which is recruited to the TNF receptor following TNFα treatment of cells, binds to IKKγ/NEMO and leads to the subsequent association of IKKα and IKKβ (37, 45).

In contrast, the association of the A20 protein with IKKγ/NEMO decreases TNFα-mediated activation of the NF-κB pathway (45). The viral transactivator Tax has also been shown to bind to IKKγ/NEMO and stimulate IKK kinase activity (42, 46, 47) as has the cellular protein CIKS (48). These results indicate that IKKγ/NEMO can interact with a variety of different regulatory proteins that are important in the activation of the NF-κB pathway in response to various stimuli.

Genetic studies have also been utilized to study the role of
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IKKγ/NEMO in regulating the NF-κB pathway (49–51). Disruption of a single copy of the IKKγ/NEMO gene, which is located on the X chromosome, results in the death of male mice in utero, while female mice develop granulocytic infiltration and both hyperproliferation and increased apoptosis of keratinocytes (49, 51). The homozygous deletion of IKKγ/NEMO results in embryonic lethality in both male and female mice due to TNFα-induced hepatic apoptosis (49, 50). Fibroblasts isolated from these mice are defective in activating the NF-κB pathway in response to a variety of stimulators of this pathway. In humans, mutation of a single copy of the IKKγ/NEMO gene is associated with a syndrome known as incontinentia pigmenti, an X-linked defect that results in lethality in males and a granulocytic infiltration of the skin in females (52). Recently, another syndrome due to mutations in the putative zinc finger domain in the C terminus of IKKγ/NEMO has been described (53–55). These mutations, which impair but do not eliminate NF-κB function, result in an X-linked immunodeficiency syndrome characterized by hyper-IgM production and hypohydror ectodermal dysplasia. Thus, both biochemical and genetic studies indicate a critical role for IKKγ/NEMO in regulating NF-κB activation.

Although IKKγ/NEMO is critical for activation of the NF-κB pathway, the exact mechanisms involved in its regulation remain to be elucidated. Previously, we demonstrated that interactions between IKKγ/NEMO and IKKβ are critical for the formation of the high molecular weight IKK complex (41). In this study, we addressed the role of IKKγ/NEMO in facilitating interactions between IκB and IKKβ. IKKγ/NEMO was critical for the association of IκB and IKKβ with the high molecular weight IKK complex. Furthermore, we found that IκB in the high molecular weight complex was preferentially phosphorylated. Finally, we demonstrated that IKKγ/NEMO enhanced the association of the IκBα and IKKβ and increased IκB kinase activity. Thus, the ability of IKKγ/NEMO to both stimulate the association of the IκB proteins with IKKβ and increase IκB kinase activity is likely important in activating the NF-κB pathway.

MATERIALS AND METHODS

DNA Constructs—The wild-type murine IKKγ/NEMO cDNA was cloned into the CMV expression vector pCMV5 fusing the Myc tag to the amino terminus of IKKγ/NEMO sequence (41). An N-terminal IKKγ/NEMO containing amino acid residues 101–412 and a C-terminal IKKγ/NEMO deletion containing amino acids 1–312 were also cloned downstream of the Myc epitope in pCMV5 (41). Wild-type human terminal IKK was isolated from these mice are defective in activating the NF-κB/H9260 pathway in response to a variety of stimulators of this pathway, the exact mechanisms involved in its regulation remain to be elucidated (41). In contrast, the N-terminal kinase activity (Fig. 1B, top panel). The same pattern of results was seen with these extracts when IKKγ was assayed with the GST-IκBα substrate (Fig. 1B, second panel). Western blot analysis confirmed the expression of the epothe-tagged IKKβ and IKKγ/NEMO proteins (Fig. 1B, third and fourth panels). These
results indicate that the N terminus of IKKγ/NEMO is critical for its ability to stimulate IKKβ phosphorylation of both IxBα and IxBβ.

IKKγ/NEMO Increases IxBα and IxBβ Association with the High Molecular Weight IKK Complex—One potential step that may be regulated by IKKγ/NEMO to activate the NF-κB pathway is to facilitate the association of IxBα and IxBβ with IKKβ in the high molecular weight IKK complex. This may lead to increased IKKβ phosphorylation of IxBα and IxBβ and thus result in enhanced IxB degradation and NF-κB nuclear translocation. Previous studies indicate that IKKα, IKKβ, and IKKγ/NEMO interact in the high molecular weight IKK complex (35, 36, 40). Our previous studies indicate that the high molecular weight IKK complex can be formed in COS cell extracts prepared following transfection of expression vectors encoding epitope-tagged IKKα, IKKβ, and IKKγ/NEMO (41). This high molecular weight complex could also be formed by transfecting IKKβ and IKKγ without IKKα and contained high levels of IKKβ kinase activity.

In order to test the possibility that IKKγ/NEMO may be involved in recruiting IxB into the high molecular weight IKK complex, expression vectors encoding FLAG-tagged IxBα, Myc-tagged IKKβ, and either wild-type Myc-tagged IKKγ/NEMO or ΔN or ΔC mutants were transfected in various combinations into COS cells (Fig. 2). S100 extracts were prepared from the transfected cells and subjected to chromatography on a Superdex 200 column in order to analyze the migration of these proteins based on their molecular mass. Western blot analysis indicated that when the FLAG-tagged IxBα protein was transfected alone, it peaked at a molecular mass of ~200 kDa (Fig. 2A). Similar migration of FLAG-tagged IxBα protein was seen when it was expressed with Myc-tagged IKKγ/NEMO (Fig. 2B). When Myc-tagged IKKβ was transfected with the FLAG-tagged IxBα, the majority of the IxBα still migrated at 200 kDa although a portion of IxBα migrated with IKKβ between 600 and 700 kDa (Fig. 2C). However, when the epitope-tagged IKKβ, IKKγ/NEMO, and IxB proteins were expressed together, a larger portion of the IxBα was found to comigrate with IKKβ and IKKγ in the high molecular weight fractions (Fig. 2D). Identical results were noted when an epitope-tagged IKKα was included with IKKβ, IKKγ/NEMO, and IxBα in these transfections (data not shown). When the C-terminal truncated form of IKKγ/NEMO was cotransfected with the FLAG-tagged IxBα and the Myc-tagged IKKβ, there was less FLAG-tagged IxBα present in the high molecular weight fractions (Fig. 2E). Transfection of the N-terminal deleted form of IKKγ/NEMO with the IxBα and IKKβ resulted in several effects. First, the majority of IKKβ migrated at a lower molecular weight than in the presence of wild-type IKKγ/NEMO (Fig. 2F). Furthermore, the majority of the IxBα protein was found to migrate at about 200 kDa, which was similar to the results seen when IxBα was transfected alone. These results suggest that IKKγ/NEMO is involved in the association of both IKKβ and IxBα in a high molecular weight IKK complex.

Next we assayed the effects of IKKγ/NEMO expression on IxBβ association with the high molecular weight IKK complex. COS cells were transfected with various combinations of FLAG-tagged IxBβ, Myc-tagged IKKβ, and wild-type or mutant Myc-tagged IKKγ/NEMO. Again S100 extracts were prepared, and following Superdex 200 chromatography, Western blot analysis was performed on the different column fractions using epitope-specific monoclonal antibodies. IxBβ was found to migrate in a very broad peak, which ranged from 200 to 500 kDa (Fig. 3A). The expression of either IKKγ/NEMO or IKKβ did not significantly alter IxBβ migration (Fig. 3, B and C). In contrast, the expression of wild-type IKKγ/NEMO with IKKβ and IxBβ resulted in the peak of both IKKβ and IxBβ shifting to 600–700 kDa (Fig. 3D). The C-terminal deleted form of IKKγ/NEMO resulted in decreased IxBβ present in the high molecular weight IKK fraction as compared with that seen with wild-type IKKγ/NEMO (Fig. 3E), while the N-terminal deleted form of IKKγ/NEMO resulted in the migration of the majority of IKKβ and IxBβ in lower molecular weight fractions (Fig. 3F). These results suggest that IKKγ/NEMO is important not only to facilitate the recruitment of IKKβ into a high molecular weight IKK complex but also to facilitate the association of IxBα and IxBβ.

IKKβ Kinase Activity Is Necessary for IxBα Recruitment by IKKγ/NEMO—Recent observations suggest that the incorporation of IKKβ into high molecular weight IKK complex by IKKγ/NEMO is not dependent on the kinase activity of IKKβ (41). To determine whether IKKγ/NEMO required an active IKKβ kinase to facilitate IxBα association, FLAG-tagged IxBα and the FLAG-tagged catalytically defective IKKβ mutant, IKKβ (K/M), were cotransfected into COS cells in the presence or absence of Myc-tagged IKKγ/NEMO. Similar to the results observed with extracts containing wild-type IKKβ and IxBα in the absence of IKKγ/NEMO (Fig. 2C), the majority of the IxBα was present in lower molecular weight fractions migrating at 200 kDa when IKKβ (K/M) was coexpressed with IxBα (Fig. 4A). However, when IKKβ (K/M) was expressed together with the epitope-tagged IKKγ/NEMO and IxBα proteins, the majority of IKKβ (K/M) but not IxBα was associated with IKKγ/NEMO in the high molecular weight fractions (Fig. 4B). These results suggest that an active IKKβ kinase is necessary for the chromatographic shift of IxBα by IKKγ/NEMO.

IxBα Association with IKKβ and IKKγ/NEMO Is Independent of Its Phosphorylation State—The previous data indicate that IKKγ/NEMO facilitates the association of both IKKβ and IxBα in a high molecular weight IKK complex and that IKKγ/NEMO facilitates recruitment of IxBα proteins. A, a schematic of the domains in the wild-type. ΔC, and ΔN IKKγ/NEMO proteins. B, an expression vector encoding FLAG-tagged IKKβ was cotransfected into COS cells with expression vectors encoding either HA-tagged wild-type, ΔC, or ΔN (aa 1–412) IKKγ/NEMO. Extracts were prepared and immunoprecipitated with the M2 monoclonal antibody, and in vitro kinase assays were performed with GST-IxBα (aa 1–54) or GST-IxBβ (aa 1–54) substrates followed by SDS-PAGE and autoradiography.

FIG. 1. IKKγ/NEMO increases IKKβ phosphorylation of the IxB proteins. A, a schematic of the domains in the wild-type. ΔC, and ΔN IKKγ/NEMO proteins. B, an expression vector encoding FLAG-tagged IKKβ was cotransfected into COS cells with expression vectors encoding either HA-tagged wild-type, ΔC, or ΔN (aa 1–412) IKKγ/NEMO. Extracts were prepared and immunoprecipitated with the M2 monoclonal antibody, and in vitro kinase assays were performed with GST-IxBα (aa 1–54) or GST-IxBβ (aa 1–54) substrates followed by SDS-PAGE and autoradiography.
NEMO requires intact IKKβ kinase activity to result in IκB association with this complex. To determine whether IκB phosphorylation was critical for its association with IKKγ/NEMO, and IKKβ in the high molecular weight fractions, a degradation-resistant IκBα mutant was transfected with wild-type IKKβ in either the presence or absence of IKKγ/NEMO. In this mutant, IκBα (SS/AA), serine residues 32 and 36, which are sites for IKKβ phosphorylation, were changed to alanine residues in order to prevent its phosphorylation and subsequent degradation in response to activators of the NF-κB pathway (33). When IκBα (SS/AA) was expressed with IKKγ, IκBα (SS/AA) was predominantly present at a molecular mass of ~200 kDa with a portion of this protein also present at 600–700 kDa (Fig. 5C). The fact that a portion of this IκB protein migrated at 600–700 kDa in the absence of transfected IKKγ/NEMO probably reflects the increased protein stability of this
mutant as compared with wild-type IxB\(\alpha\) and its binding to low levels of endogenous IKK\(\gamma\)/NEMO. The expression of IKK\(\gamma\)/NEMO with IKK\(\beta\) and IxB\(\alpha\) resulted in the peak of IxB\(\alpha\) (SS/AA) shifting to between 600 and 700 kDa (Fig. 5D). This result is similar to the effects of IKK\(\gamma\)/NEMO that were seen when it was expressed with IKK\(\beta\) and wild-type IxB\(\alpha\) (Fig. 5, A and B). Taken together, these results suggest that IxB\(\alpha\) association in the high molecular weight complex is not dependent on its N-terminal phosphorylation, but it does require IKK\(\gamma\)/NEMO and active IKK\(\beta\).

**IKK\(\gamma\)/NEMO Facilitates IKK\(\beta\) Association with IxB\(\alpha\) and IxB\(\beta\).** In order to further explore the role of IKK\(\gamma\)/NEMO on facilitating interactions between IKK\(\beta\) and IxB\(\alpha\), expression vectors encoding FLAG-tagged IxB\(\alpha\), HA-tagged IKK\(\beta\), and Myc-IKK\(\gamma\)/NEMO were transfected in various combinations into COS cells. Following immunoprecipitation of either HA-IKK\(\beta\) or Myc-IKK\(\gamma\)/NEMO, Western blot analysis with epitope-specific antibodies directed against either IxB\(\alpha\), IKK\(\gamma\), or IKK\(\beta\) was performed (Fig. 6A). Immunoprecipitation of extracts transfected with FLAG-IxB\(\alpha\) and Myc-IKK\(\gamma\)/NEMO with the 12CA5 antibody directed against the HA-epitope indicated that there was little nonspecific association of FLAG-IxB\(\alpha\) (Fig. 6A, lane 2, top panel). When FLAG-IxB\(\alpha\) and HA-IKK\(\beta\) were co-transfected, immunoprecipitation of the HA-IKK\(\beta\) resulted in the association of a small amount of FLAG-IxB\(\alpha\) (Fig. 6A, lane 3, top panel). However, transfection of Myc-IKK\(\gamma\)/NEMO, FLAG-IxB\(\alpha\), and HA-IKK\(\beta\) resulted in the increased association of FLAG-IxB\(\alpha\) with the immunoprecipitated HA-IKK\(\beta\) (Fig. 6A, lane 4, top panel). Transfection of a C-terminal truncated form of Myc-IKK\(\gamma\) also resulted in increased association of FLAG-IxB\(\alpha\) with the immunoprecipitated HA-IKK\(\beta\) (Fig. 6A, lane 5, top panel). Transfection of an N-terminal truncated form of Myc-IKK\(\gamma\) did not significantly increase FLAG-IxB\(\alpha\) association with HA-IKK\(\beta\) (Fig. 6A, lane 6, top panel). Both the wild-type and the C-terminal deletion of IKK\(\gamma\)/NEMO, but not the N-terminal deletion, communoprecipitated with IKK\(\beta\) and IxB\(\alpha\) (Fig. 6A, second panel, lanes 4–6). These results suggest that IKK\(\beta\), IKK\(\gamma\)/NEMO, and IxB\(\alpha\) form a complex.

The Myc antibody was next used to immunoprecipitate the Myc-IKK\(\gamma\)/NEMO protein from these extracts (Fig. 6A, third panel). IKK\(\gamma\)/NEMO alone did not result in the association of IxB\(\alpha\) (Fig. 6A, lane 2, third panel). However, the presence of either the wild-type or the C-terminal truncation of IKK\(\gamma\)/NEMO, but not the N-terminal truncation, resulted in the association of both IxB\(\alpha\) and HA-IKK\(\beta\) (Fig. 6A, lanes 4–6, third and fourth panels). Western blot analysis demonstrated the expression of each of these proteins following transfection (Fig. 6A, bottom three panels). These results also indicate that the expression of IKK\(\gamma\)/NEMO with IKK\(\beta\) increases the association of IxB\(\alpha\).

Similar transfection assays were performed with FLAG-IxB\(\beta\), HA-IKK\(\beta\), and Myc-IKK\(\gamma\)/NEMO in order to determine the role of IKK\(\gamma\)/NEMO and IKK\(\beta\) in the association with IxB\(\beta\) (Fig. 6B). When antibody to the HA epitope was used in the immunoprecipitation assays, there was little nonspecific association of IxB\(\beta\) with IKK\(\gamma\)/NEMO in the absence of HA-tagged IKK\(\beta\) (Fig. 6B, lane 2, top panel). FLAG-IxB\(\beta\) was able to associate with HA-IKK\(\beta\) alone (Fig. 6B, lane 3, top panel), and this association was increased following the expression of Myc-IKK\(\gamma\)/NEMO (Fig. 6B, lane 4, top panel). The expression of either the N- or C-terminal IKK\(\gamma\)/NEMO constructs resulted in less association of FLAG-IxB\(\beta\) with HA-IKK\(\beta\) than did wild-
FIG. 6. IKKγ/NEMO facilitates the interactions of IκBα and IKKB. Expression vectors (3 μg) encoding either FLAG-tagged IκBα (A) or FLAG-tagged IκBβ (B) were co-transfected into COS cells (107 cells/100-mm plate) with an HA-tagged IKKB expression vector and either Myc-tagged wild-type (WT) or mutated (ΔC or ΔN) expression vectors encoding IKKγ/NEMO as indicated. At 36 h post-transfection, the cells were harvested, and equal amounts of protein from the S100 extracts were immunoprecipitated with antibodies directed against either the HA (IKKB) or Myc (IKKγ) epitopes (middle two panels), and the amounts of the associated proteins were determined by immunoblotting. The expression level of IκBα, IKKB, and IKKγ/NEMO in the lysates before immunoprecipitation was determined by immunoblotting with antibodies directed against FLAG, HA, or Myc, respectively (lower three panels).

A. 

B.

Type IKKγ/NEMO (Fig. 6B, lanes 5 and 6, top panel). The wild-type and C-terminal deletion of IKKγ/NEMO, but not the N-terminal deletion, also co-immunoprecipitated with IKKB and IκBβ (Fig. 6B, lanes 4–6, second panel).

Next, immunoprecipitation of Myc-IKKγ/NEMO was performed. In extracts containing wild-type Myc-IKKγ/NEMO and FLAG-IκBβ, immunoprecipitation revealed no association of IκBβ with IKKB (Fig. 6B, lane 3, third panel). In the presence of wild-type Myc-IKKγ/NEMO, HA-IKKB, and FLAG-IκBβ, both IKKB and IκBβ were immunoprecipitated with Myc-IKKγ antibody (Fig. 6B, lane 4, third panel). The expression of either the C-terminal or N-terminal IKKγ/NEMO deletions with IKKB and IκBβ resulted in decreased association of IκBβ (Fig. 6B, lanes 5 and 6, third panel). IKKB associated with both the wild-type and the C-terminal truncation of IKKγ/NEMO but not the N-terminal truncation (Fig. 6B, lanes 4–6, fourth panel). Western blot analysis of these extracts demonstrated the expression of these proteins (Fig. 6B, bottom three panels). These results indicate that IKKγ/NEMO increases the association of both IκBα and IκBβ with IKKB.

TNFa Treatment Increases the Amount of Phosphorylated IκBα in the IKK Complex—The previous studies were performed following transfection of wild-type and mutant IκBα, IKKB, and IKKγ/NEMO proteins to characterize the roles of each of these proteins in the assembly of the IKK complex. In order to further address the relevance of these observations, we next characterized the components of the endogenous IKK complex following chromatographic fractionation of S100 extracts prepared from untreated and TNFa-treated HeLa cells (Fig. 7). Western blot analysis indicated that the majority of the endogenous IKKB and IKKγ/NEMO proteins were present in a complex migrating between 600 and 700 kDa in extracts prepared from both untreated and TNFa-treated HeLa cells (Fig. 7A). In addition to this peak, a smaller amount of IKKγ/NEMO was also detected in a lower molecular weight complex migrating between 200 and 300 kDa (Fig. 7A). Quantitation of these gels by densitometry indicated that more than 50% of the IKKB and IKKγ/NEMO proteins were present in column fractions 8 and 9. In extracts prepared from unstimulated HeLa cells, the peak of endogenous IκBα was found to migrate at ~500 kDa. However, IκBα was not detected in extracts prepared from TNFa-stimulated cells using either a rabbit polyclonal IκBα antibody or a phosphospecific IκBα antibody that recognized IκBα phosphorylated on serine residue 32 (Fig. 7A). IKK kinase activity was assayed using a GST-IκBα substrate and was found to be maximal in column fractions 8 and 9 that contained the majority of the IKKB and IKKγ/NEMO proteins (Fig. 7A).

In an attempt to analyze the presence of the phosphorylated form of IκBα following TNFa stimulation, the proteasome inhibitor MG-132 was added to HeLa cells for 1 h prior to treatment with TNFa in order to prevent IκBα degradation by the proteasome (Fig. 7B). The addition of MG-132 did not change the distribution of IKKB and IKKγ/NEMO with more than 60% of these proteins present in column fractions 8 and 9 in extracts prepared from untreated and TNFa-treated HeLa cells (Fig. 7B). However, in the presence of MG-132 there was a 4-fold increase in the amount of phosphorylated IκBα in TNFa-treated HeLa extracts. Nearly 50% of this phosphorylated form of endogenous IκBα was detected in column fractions 8 and 9 versus only 30% in extracts prepared in the absence of TNFa (Fig. 7B). The presence of phosphorylated IκBα correlated with column fractions that contained increased IKK activity, suggesting that the presence of IKKB, IKKγ/NEMO, and IκBα in the high molecular weight complex correlated with TNFa-induced increases in IκBα phosphorylation.

Similar experiments were also performed to determine the distribution of IKKB, IKKγ/NEMO, and IκBα in extracts prepared from untreated and TNFa-treated parental mouse embryonic fibroblasts (MEF) and IKKγ/NEMO-deficient cells (Fig. 8). As expected, based on our previous results, there was a 4-fold increase in the amount of phosphorylated IκBα in column fractions 8 and 9 in extracts prepared from TNFa-treated MEF cells versus only 30% in extracts prepared from untreated MEF cells. There was no change in the distribution of either IKKB or IKKγ/NEMO following TNFa-treatment (Fig. 8A). Interestingly, in extracts prepared from MEF cells, the majority of IKKB was found to migrate at ~200 kDa, while only a small portion of IKKB was detected at 600–700 kDa. Furthermore, IKKB migrated in this high mo-
Fig. 7. TNFα treatment increases the levels of phosphorylated IκBα in the IKK complex. HeLa cells were either not treated (top panel) or treated with TNFα (20 ng/ml) (lower panel) for 20 min in the absence (A) or presence (B) of MG-132 (25 μM). S100 extracts were prepared from these cells and fractionated by chromatography on a Superdex-200 column. Proteins were analyzed by Western blotting using antibodies directed against IκBα (Santa Cruz Biotechnology; sc-7607), or IKKγ/NEMO (Santa Cruz Biotechnology; sc-8330). Western blots were analyzed by densitometry (Bio-Rad Molecular Analysis II). In vitro kinase activity was analyzed utilizing a GST/IκBα (aa 1–54) substrate following immunoprecipitation of the each fractions with IKKα (sc-7606) antibody. The positions of the molecular weight markers and the numbers of the column fractions are indicated.

Fig. 8. Chromatographic distribution of IKK components in extracts prepared from MEF cells and IKKγ/NEMO-deficient cells. Either parental MEFs (A) or MEFs that contained a homozygous deletion of the IKKγ/NEMO genes (B) were untreated (top panel) or treated with TNFα (20 ng/ml) (bottom panel) for 20 min in the presence of MG-132 (25 μM). S100 extracts were prepared from these cells and fractionated by chromatography on a Superdex-200. Proteins in each fraction were analyzed by Western blotting, an in vitro kinase assay was performed as described in the legend to Fig. 7, and densitometry was performed. IKK kinase activity was assayed in each column fraction using the GST-IκBα substrate. The positions of the molecular weight markers and the numbers of the column fractions are indicated.

Increasing Amounts of IKKγ/NEMO Facilitate IKKβ Association with IκBα and Increased IKKβ Kinase Activity—The results presented suggested that IKKγ/NEMO facilitates the association of IKKβ and IκBα and that this process was important in the ability of IKKγ/NEMO to stimulate IKKβ phosphorylation of IκBα. Next we determined whether the transfection of increasing amounts of IKKγ/NEMO would increase the association of IκBα and IKKβ and stimulate IKKβ kinase activity. Expression vectors encoding Myc-tagged IKKβ and a FLAG-tagged IκBα (SS/AA) mutant were transfected into COS cells with increasing concentrations of an HA-tagged IKKγ/NEMO expression vector. This IκBα mutant was utilized in these studies in order to quantitate IκBα association in the absence of potential IKKβ-mediated degradation. S100 extracts were prepared, and a monoclonal antibody directed against the Myc epitope was used to immunoprecipitate IKKβ followed by Western blot analysis with the M2 monoclonal antibody directed against the FLAG-tagged IκBα mutant (Fig. 9A). These results demonstrated that the expression of increasing amounts of IKKγ/NEMO resulted in enhanced association of IκBα with IKKβ (Fig. 9A). Densitometry revealed a 6-fold
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**Fig. 9.** IKKγ/NEMO leads to the association of IκBα and IKKβ and increases IκBα phosphorylation. A, an expression vector encoding the FLAG-tagged IκBα (SS/AA) (2 μg) was co-transfected into COS cells with a Myc-tagged IKKγ expression vector (2 μg) and either no (lane 1) or increasing amounts of an HA-tagged IKKγ/NEMO expression vector, 0.02 μg (lane 2), 0.2 μg (lane 3), 2 μg (lane 4), or 4 μg (lane 5). The CMV expression vector alone was used to standardize the amount of DNA in each transfection. Equal amounts of S100 extract (200 μg) prepared from the COS cells were immunoprecipitated with antibodies directed against Myc (IKKγ), and the amount of the associated IκBα (SS/AA) proteins was determined by immunoblotting with a monoclonal antibody (M2) directed against the FLAG epitope. The expression level of IκBα (SS/AA), IKKγ, and IKKγ/NEMO in the lysates before immunoprecipitation was determined by immunoblotting with monoclonal antibodies directed against FLAG, Myc or HA, respectively. The percentage of IκBα that was immunoprecipitated based on densitometry of the Western blot analysis performed on both IκBα present in S100 and immunoprecipitated extracts was 6.9% (lane 1), 11.5% (lane 2), 22.6% (lane 3), 46.1% (lane 4), and 32.8% (lane 5). B, COS cells were transfected with a CMV expression vector alone (lane 1), IKKγ alone (lane 2), or IKKγ and increasing amounts of IKKγ/NEMO, 0.005 μg (lane 3), 0.05 μg (lane 4), 0.25 μg (lane 5), or 0.5 μg (lane 6). In vitro IKKα kinase activity was assayed following immunoprecipitation with FLAG (IKKγ) antibody utilizing the GST/IκBα (aa 1–54) substrate, and autoradiography was performed. The relative IKKα kinase activity as determined by PhosphorImager analysis in this experiment was 1.0 (lane 1), 1.2 (lane 2), 1.1 (lane 3), 5.0 (lane 4), 12.5 (lane 5), and 18.0 (lane 6).

increase in the amount of IκBα associated with IKKβ following the transfection of increasing concentrations of IKKγ/NEMO. Western blot analysis revealed that the levels of the epitope-tagged IKKβ and IκBα in the S100 extracts were unchanged when increasing amounts of IKKγ/NEMO were transfected (Fig. 9A).

Next the ability of increasing amounts of IKKγ/NEMO to stimulate IKKβ kinase activity was determined. An IKKγ expression vector and increasing amounts of an expression vector encoding IKKγ/NEMO were transfected into COS cells. S100 extracts were prepared and immunoprecipitated with the Myc antibody that recognizes the Myc-tagged IKKβ. In vitro kinase assays with a GST-IκBα substrate were then performed and indicated that transfection of increasing concentrations of IKKγ/NEMO stimulated IKKβ phosphorylation of the IκBα substrate ~18-fold (Fig. 9B). These results suggest that IKKγ/NEMO can both facilitate the association of IκBα with IKKβ and increase IKKβ kinase activity for the IκBα substrate.

**DISCUSSION**

IKKγ/NEMO is essential for the activation of the NF-κB pathway in response to a variety of stimuli (34–41, 49–51). For example, cell lines deficient in IKKγ/NEMO expression are defective in NF-κB activation (34, 49–51). The defects in NF-κB signaling in the absence of IKKγ/NEMO probably result from two defects. First, the high molecular weight IKK complex cannot be assembled in the absence of IKKγ/NEMO (34, 49–51). Second, IKKγ/NEMO is probably critical for the recruitment of upstream kinases, such as RIP, that are involved in stimulating IKK activity (37, 45) and for the interaction with IKKα and IKKβ (48). In this study, we further explore the mechanisms by which IKKγ/NEMO stimulates the NF-κB pathway and demonstrate that IKKγ/NEMO also plays a role in the association of IKKβ with the IκB proteins in the high molecular weight IKK complex and increases IKKβ activity to result in enhanced IκB phosphorylation.

Both wild-type and a C-terminal deleted form of IKKγ/NEMO increased IKKβ phosphorylation of IκBα and IκBβ in in vitro kinase assays. However, an N-terminal deleted form of IKKγ/NEMO that is unable to bind to IKKβ (12, 36, 41, 42) did not stimulate IKKβ phosphorylation of the IκB proteins. These results indicate that IKKγ/NEMO binding to IKKβ correlates with increases in IKKβ-mediated phosphorylation of the IκB.

Whether the effect of IKKγ/NEMO was due strictly to a catalytic effect on IKKβ or potentially to IKKβ/NEMO enhancement of IKKβ binding to IκB was next addressed.

Previous results suggest that IKKγ/NEMO interacts predominately with IKKβ rather than IKKα to assemble the high molecular weight IKK complex (34, 36, 41). Since IKKβ is the critical kinase involved in activating the NF-κB pathway (17–22, 59), it was important to address whether IKKγ/NEMO could facilitate the association of the IκB proteins with IKKβ in the high molecular weight IKK complex. We found that, following transfection of expression vectors encoding IKKβ, IKKγ/NEMO, and IκBα, IKKβ itself could result in limited association of the IκB proteins in the high molecular weight fractions obtained following column chromatography. In contrast, IKKγ/NEMO alone did not result in enhanced IκB association with the high molecular weight IKK complex. However, the presence of both IKKγ/NEMO and IKKβ significantly enhanced the association of the IκB proteins in the high molecular weight fractions. In contrast, an N-terminal truncation of IKKγ/NEMO that did not bind to IKKβ was unable to enhance IκB association into the high molecular weight fractions. These results indicate that the presence of IKKγ/NEMO results in the association of both IκBα and IKKβ in the high molecular weight complex.

Next we investigated the requirements of IKKγ/NEMO to facilitate IκB association with the high molecular fractions containing IKK. IKKγ/NEMO could recruit a kinase-dead IKKβ mutant into the high molecular weight fractions, but IKKγ/NEMO and this mutant did not facilitate IκB association. However, a degradation-resistant IκB mutant could associate with IKKβ and IKKγ/NEMO in the high molecular weight fractions, suggesting that phosphorylation of IκB is not a requirement for its association with the high molecular weight IKK complex. Thus, IKKγ/NEMO and an active IKKβ kinase are required for optimal association of IκB in the high molecular weight IKK complex.

Analysis of column chromatography of extracts prepared from untreated and TNFα-treated HeLa, MEF, and IKKγ/NEMO knock-out cells was performed. These studies indicated that TNFα treatment results in marked increases in the amount of phosphorylated IκBα in the high molecular weight fractions containing IKKγ/NEMO and IKKβ. Moreover, the
presence of phosphorylated IkBa in these fractions correlated with TNFα-mediated increases in IkB kinase activity. Cells lacking IKK/NEMO did not generate a high molecular weight IKK complex, and phosphorylated IkBa was not detected. These results indicate that the high molecular weight complex that contains IKK/NEMO, IKKβ, and IkB is the target for TNFα-mediated increases in IkB kinase activity and IkBa phosphorylation. However, we cannot state conclusively that IkBa is preferentially phosphorylated in the high molecular weight complex.

Finally, coimmunoprecipitation studies were performed to assay for the in vivo interactions between IKK/NEMO, IKKβ, and IkB. Although the IkB proteins could associate with IKKβ alone, they could not associate with IKK/NEMO alone. However, these studies demonstrate that the presence of IKKγ/NEMO could enhance the association of IKKβ with the IkB proteins. Furthermore, transfection of increasing amounts of IKK/NEMO resulted in enhanced association of IKKβ with IkBa and increased IKKβ kinase activity. These results suggest that IKKγ/NEMO probably has at least two major functions. First, it stimulates IKKβ kinase activity either by stimulating IKK autoposphorylation or by recruitment of upstream kinases (37, 45). Second, it recruits the IKK proteins into a high molecular weight complex and facilitates the interactions between IKKβ and IkBa. Thus, IKKγ/NEMO appears to function as an adaptor protein to increase the interactions of key factors required for NF-κB activation.

The mechanisms by which IKKγ/NEMO leads to recruitment of the IkBα proteins into the high molecular weight complex remains to be determined. IKKγ/NEMO binding to IKKβ may alter IKKβ conformation to enhance the association of the IkBα and IkBβ substrates in the high molecular weight IKK complex. Alternatively, IKKγ/NEMO and IKKβ may both directly interact with IkBα. Finally, it is possible that IKKγ/NEMO recruits additional cellular proteins, which are required to facilitate the interactions between IKKβ and IkBα. The enhanced association of the IkBα proteins and IKKβ, which is mediated by IKKγ/NEMO, probably results in the increased phosphorylation of IkBa in response to various activators of the NF-κB pathway. Consistent with these findings, TNFα treatment did not alter IkBα migration following chromatography of extracts prepared from IKKγ/NEMO-deficient cells. Thus, one defect in activating the NF-κB pathway in the absence of the IKKγ/NEMO proteins may be due to the failure of IKKβ to associate with the IkBα proteins to enhance its phosphorylation and degradation. Another defect in cells lacking IKKγ/NEMO is probably due to the failure to activate IKKβ kinase activity. Further studies will be necessary to better define the interactions of these proteins and how they lead to activation of the NF-κB pathway.
IKK\(\gamma\)/NEMO Facilitates Recruitment of I\(\kappa\)B Proteins

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