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NIBP, a Novel NIK and IKKβ-binding Protein That Enhances NF-κB Activation*

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Wen-Hui Hu‡§¶‖, Julie S. Pendergast‡¶, Xian-Ming Mo‡‡, Roberta Brambilla‡, Valerie Bracchi-Ricard‡, Fang Li‡, Winston M. Walters‡, Bas Blits‡, Li He‡, Sandra M. Schaal‡**, and John R. Bethea‡**§§**

*From ‡The Miami Project to Cure Paralysis, Department of Neurological Surgery, Miller School of Medicine, University of Miami, Miami, Florida 33136, the **Neuroscience Program, Miller School of Medicine, University of Miami, Miami, Florida 33136, the ‡‡Laboratory of Hematology, West China Hospital, Sichuan University, Chengdu 610041, China, and the §Department of Physiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298*

The transcription factor NF-κB plays an important role in both physiological and pathological events in the central nervous system. Nevertheless, the mechanisms of NF-κB-mediated regulation of gene expression, and the signaling molecules participating in the NF-κB pathway in the central nervous system are, to date, poorly understood. To identify such molecules, we conducted a yeast two-hybrid screen of a human brain cDNA library using NIK as bait. As a result, we identified a novel NIK and IKKβ binding protein designated NIBP that is mainly expressed in brain, muscle, heart, and kidney. Interestingly, low levels of expression were detected in immune tissues such as spleen, thymus, and peripheral blood leukocytes, where NF-κB is known to modulate immune function. We demonstrated by immunohistochemistry that NIBP expression in the brain is localized to neurons. NIBP physically interacts with NIK, IKKβ, but not IKKα or IKKγ. NIBP overexpression potentiates tumor necrosis factor-α-induced NF-κB activation through increased phosphorylation of the IKK complex and its downstream IκBα and p65 substrates. Finally, knockdown of NIBP expression by small interfering RNA reduces tumor necrosis factor-α-induced NF-κB activation, prevents nerve growth factor-induced neuronal differentiation, and decreases Bcl-xL gene expression in PC12 cells. Our data demonstrate that NIBP, by interacting with NIK and IKKβ, is a new enhancer of the cytokine-induced NF-κB signaling pathway. Because of its neuronal expression, we propose that NIBP may be a potential target for modulating the NF-κB signaling cascade in neuronal pathologies dependent upon abnormal activation of this pathway.

NF-κB is a multipotent transcription factor that regulates the expression of numerous genes involved in a wide array of biological responses such as inflammation, immunity, apoptosis, and synaptic plasticity (1–4). In mammals, the NF-κB family of transcription factors consists of five members: p65 (or RelA), RelB, c-Rel, NF-κB1 (or p105, precursor of p50), and NF-κB2 (or p100, precursor of p52). To date, two principal pathways for NF-κB activation have been characterized, a classical and an alternative pathway (5–7). The classical pathway is triggered by stimuli such as tumor necrosis factor α (TNFα),[1] IL-1, CD40 ligand, and lymphotoxin-β (8). Activation of this pathway depends on the IκB kinase (IKK) signalsome, which consists of at least two catalytic subunits (IKKα or IKK1, and IKKβ or IKK2) and a regulatory subunit (IKKγ or NEMO). The activated IKK complex phosphorylates the inhibitor proteins of NF-κB (IκBs) to induce their ubiquitination and degradation, resulting in the translocation of NF-κB dimers (mainly p65/p50) to the nucleus and transcriptional activation of specific target genes. This pathway is crucial for the activation of innate immunity and inflammation. The alternative pathway is activated by lymphotoxin-β (8–10), CD40 ligand (11), and B cell-activating factor (12). It relies on the phosphorylation of IKKα homodimers by NF-κB inducing kinase (NIK) to induce p100 processing and nuclear translocation of RelB/p52 dimers (7). This alternative pathway is necessary for secondary lymphoid organ development, maturation of B cells, and adaptive humoral immunity.

NF-κB is involved in the regulation of both physiological and pathological processes. Synaptic stimulation activates NF-κB, which then participates in long-term potentiation, a process associated with memory formation (13). In addition, NF-κB has anti-apoptotic properties in neurons because blocking its activation increases their sensitivity to TNFα-induced apoptosis (14, 15). Induction of NF-κB is also associated with several neurological diseases such as Parkinsons (16), Alzheimers (17), AIDS dementia (18, 19), and spinal cord injury (20, 21). NIK also appears to perform specific functions in the central nervous system and has been implicated in the activation of both the classical and alternative pathways.

NIK was originally identified as a serine/threonine protein kinase related to the mitogen-activated protein kinase (MAP3K) that interacts with TNF receptor-associated factor 2 (TRAF2), IKKα, IKKβ, and can strongly activate NF-κB follow-

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¶ Both are considered co-first authors.

‖ To whom correspondence may be addressed: Dept. of Physiology, Medical College of Virginia, VA Commonwealth University, Sanger Hall, Rm. 12-002, 1101 East Marshall St., Richmond, VA 23298. Tel.: 804-828-8504; Fax: 804-828-2500; E-mail: whu@vcu.edu.

§§ To whom correspondence may be addressed: The Miami Project To Cure paralysis, Miller School of Medicine, University of Miami, 1095 NW 14th Terrace, Miami, FL 33136. Tel.: 305-243-3804; Fax: 305-243-3914; E-mail: JBethea@miami.edu.

[1] The abbreviations used are: TNFα, tumor necrosis factor α; NF-κB, nuclear factor κB; NIK, NF-κB inducing kinase; IKK, IκB kinase; IL-1, interleukin-1; TRAF2, tumor necrosis factor receptor-associated factor-2; NGF, nerve growth factor; GST, glutathione *S*-transferase; siRNA, small interfering RNA; MAP, mitogen-activated protein.

ing TNF/NGF receptor family stimulation (22). Although earlier overexpression studies using mutant NIK pointed to a crucial role for NIK in TNFα-induced NF-κB activation, later studies using NIK knock-out mice challenged this view, finding NIK to be an essential component only of the alternative pathway. More recent data, however, indicated that NIK participates in signaling events initiated by specific inducers that activate both the classical and alternative pathways (23).

The first demonstration of the function of NIK in the central nervous system was the observation that *Aly* mice, in which a naturally occurring mutation in the alymphoplasia allele causes NIK to be non-functional, displayed progressive neurological abnormalities leading to hind limb paralysis (24). Moreover, in PC12 cells, NIK promotes neurite formation and prevents apoptosis (25). Nevertheless, the regulatory mechanisms of NIK and, in general, of the NF-κB signaling pathway in the central nervous system are not well understood. To investigate these mechanisms, we performed a yeast two-hybrid screen of a brain cDNA library with NIK as bait. As a result, we identified a novel protein designated NIBP (for NIK and IKKβ binding protein), which interacts directly with NIK and IKKβ and is expressed in neurons. NIBP functions as an enhancer of cytokine-mediated NF-κB activation and IKK kinase activity. Finally, preliminary *in vitro* studies suggest that NIBP may be a regulator of *Bcl-xL* gene expression as well as neuronal differentiation, because it is required for NGF-induced neurite extension in PC12 cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Yeast two-hybrid assay was performed with the Matchmaker Two-hybrid System II (Clontech, Palo Alto, CA). The bait, encoding the N-terminal domain (amino acids 1–145) of NIK, was inserted into the GAL4 DNA binding domain in the yeast expression vector pBridge (Clontech) as previously described (26). The yeast strain G1945 was transformed with pBridge-NIK followed by the pACT2 expression vector that contained a human brain cDNA library fused to the GAL4 transactivation domain.

Reagents and Antibodies—Human recombinant TNFα, IL-1β, IFN-γ, and NGF as well as antibodies against MAP2, FLAG, β-actin, and β-tubulin were purchased from Sigma. Antibodies against phospho-IKKα/β, phospho-p65, phospho-IκBα, NIK, and p65 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against IKKα/β, IKKα, NIK, and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A peptide corresponding to amino acids ^{417}VYNPMPFELRVENMGLLTSGVEF^{439} of NIBP (100% homology between human, mouse, and rat) was used as an immunogen to generate polyclonal antiserum in rabbit. The crude NIBP antiserum was affinity-purified (Proteintech Group, Inc.) and its specificity was verified by enzyme-linked immunosorbent assay, immunoblotting, immunoprecipitation, and immunohistochemistry.

Expression Vectors—The *NF-κB*-luciferase and *interferon response factor-1*-luciferase reporter constructs were obtained from Clontech (Palo Alto, CA). Mammalian expression vectors encoding *TRAF2*, *NIK*, *IKKα*, *IKKβ*, *IKKγ*, and Rous sarcoma virus-*β-galactosidase* were previously described (27–29). *NIBP* was generated by conventional PCR from a mouse cDNA library and cloned into the pCMV-Tag 2B mammalian expression vector (Stratagene, La Jolla, CA). The C-terminal portion (211 amino acids) of *NIBP* was generated by PCR and cloned into a cytomegalovirus promoter-based pRK7 vector (28).

Northern Blot—Tissue-specific expression of *NIBP* mRNA was examined by hybridization of a human multiple tissue Northern blot containing 2 μg of poly(A)$^+$ RNA (Clontech) with a PCR-produced, [^{32}P]dCTP-labeled 630-bp probe. Hybridizations were conducted in the ExpressHyb hybridization solution (Clontech) at 65 °C for 2 h according to the manufacturer's protocol. The same blots were then stripped and hybridized with a ^{32}P-labeled 300-bp *β-actin* probe as control.

Immunohistochemistry—Fifteen-μm cryostat sections from mouse brain and spinal cord were incubated overnight with anti-NIBP (1:1000). Antibody labeling was visualized with 3,3′-diaminobenzidine staining using the Vector Elite ABC kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions.

Cell Culture and Transfection—PC12 cells (ATCC, Manassas, VA) were cultured in minimal essential medium containing 10% horse serum, 5% fetal bovine serum (Hyclone, Logan, UT), and penicillin/streptomycin (100 units/ml). HEK293T cells (Clontech) were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml). Mouse primary cortical neurons were prepared as previously described (30). Ten-cm dishes containing 3×10^6 HEK293 cells were transiently transfected with 10 μg of plasmid DNA by the calcium phosphate precipitation technique.

Glutathione S-Transferase (GST) Pull-down Assay—The C-terminal portion (211 amino acids) of *NIBP* was cloned into the pGEX-4T-2 expression vector (Amersham Biosciences) to generate a GST-NIBP(cd) fusion protein, which was incubated with *in vitro* translated NIK, IKKα, IKKγ, and truncated IKKβ. *In vitro* translation was performed with the TNT® System (Promega, Madison, WI) in the presence of [^{35}S]methionine. Truncated IKKβ (288 amino acids from the C terminus) was generated by EcoRI digestion. *In vitro* translation mixture was incubated overnight at 4 °C with bacterially expressed GST fusion proteins coupled to 20 μl of glutathione-Sepharose beads. Following multiple washes (wash buffer: 20 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5% Nonidet P-40), GST-bound proteins were eluted with SDS-PAGE sample buffer, resolved on a 10% polyacrylamide gel, and detected by autoradiography.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and Western blot experiments were performed according to standard procedures as previously described (31).

Reporter Gene Assay—The chemiluminescent reporter gene assay for the combined detection of luciferase and β-galactosidase activity was performed with the Dual-Light Combined Reporter Gene Assay System from Applied Biosystems (Foster City, CA) according to the manufacturer's protocol. Luciferase activity was normalized to β-galactosidase. Four separate experiments were conducted and, in each experiment, data were calculated as the average ± S.E. of triplicate samples.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared according to Dignam *et al.* (32). Electrophoretic mobility shift assays were carried out as previously described (20). Briefly, double-stranded NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) were ^{32}P-labeled with T4 polynucleotide kinase (Promega), and added to binding reactions in the presence of poly(dI-dC):poly(dI-dC) (Sigma), herring sperm DNA (Invitrogen), and nuclear extracts. Equal amounts of extracts, varying from 5 to 20 μg/sample, were loaded in each binding reaction. After a 30-min incubation at room temperature, samples were loaded onto a pre-electrophoresed 0.5× Tris-borate EDTA buffer, 6% polyacrylamide gel, and run at 150 V for ~1.5 h. Gels were then fixed and dried, and autoradiograms obtained.

Lentiviral siRNA Vector and Infection—Lentiviral-mediated U6-promoted *NIBP* siRNA constructs were generated using a modified PCR-based strategy (33, 34). Sense and antisense *NIBP* oligonucleotides were cloned into the XbaI/XhoI site of the pLL3.7 vector (a kind gift from Dr. Van Parijs Lab, MIT Center for Cancer Research). Three *NIBP* siRNA lentiviral vectors containing nucleotides 586–607 (NR), 1762–1784 (MR), and 2303–2321 (CR) of mouse *NIBP* (GenBank™ accession number AY630620) were generated. Packaging, purification, and determination of virus titer was performed as described (33).

PC12 Cell Differentiation—PC12 cells were infected with siRNA lentivirus for 4 weeks prior to differentiation experiments. Cells were plated on collagen-coated 24-well dishes at a density of 10^4 cells/well. Twenty-four hours later, cells were treated with NGF (100 ng/ml). Three days after treatment, the fluorescence of enhanced green fluorescent protein in infected cells was observed.

Reverse Transcriptase-PCR—Total RNA was isolated with the TRIzol reagent (Invitrogen). Two micrograms of DNase I-treated RNA was used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen) with random hexanucleotide primers. PCR was performed on the cDNA using specific primers for *Bcl-xL* (sense, 5′-GGAGAGCGTTCAGTGATC-3′ and antisense, 5′-CAATGGTGGCTGAAGAGA-3′) and glyceraldehyde-3-phosphate dehydrogenase (sense, 5′-CTCGTGGTTCACACCCAT-3′ and antisense, 5′-GGCTGCCTTCTCTTGTGA-3′) (35).

RESULTS

Cloning and Characterization of NIBP—To investigate the regulatory mechanisms controlling NF-κB signaling in the central nervous system, we performed a yeast two-hybrid screen of a human brain cDNA library using as bait for the N-terminal portion of NIK (amino acids 1–145). From 3×10^6 yeast transformants, 40 clones were both histidine and β-galactosidase

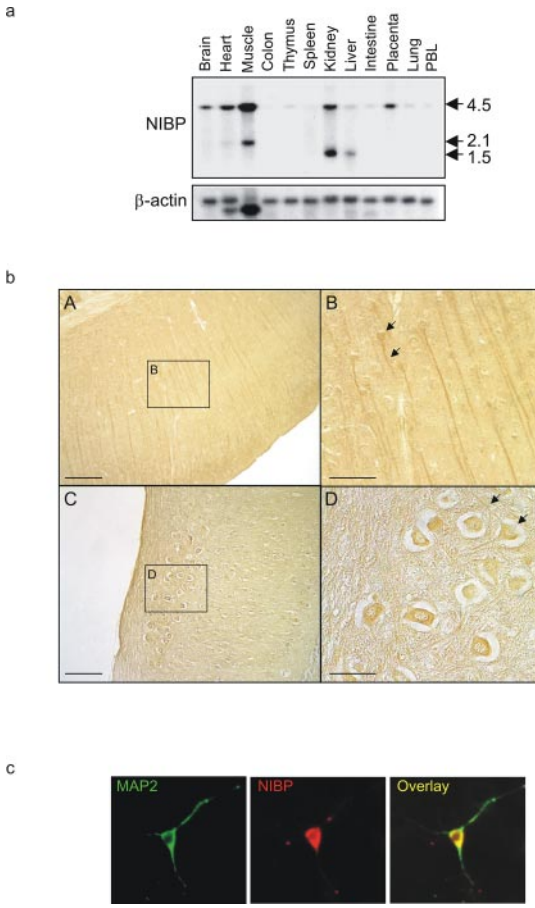

FIG. 1. **NIBP expression and tissue distribution.** *a*, *NIBP* expression profile in human tissue. A human multiple tissue mRNA blot was hybridized with a ^{32}P-labeled *NIBP* probe. A predominant transcript was ubiquitously detected at 4.5 kb. In addition, two smaller transcripts were detected in selected tissues at 2.1 (muscle and heart) and 1.5 kb (kidney and liver). In the *bottom panel*, the blot was hybridized with a β-actin probe as control. *b*, NIBP localization in the central nervous system. Mouse brain and spinal cord sections were immunolabeled with an anti-NIBP antibody. Intense 3,3′-diaminobenzidine positive neurons were detected in the pyramidal layer of the frontal cortex (*A* and *B*); 3,3′-diaminobenzidine-positive motorneurons were also observed in the ventral horns of the spinal cord (*C* and *D*). NIBP labeling was localized to both cell bodies and processes (*arrows*). *A* and *C*, *scale bar*: 120 μm. *B* and *D*, *scale bar*: 20 μm. *c*, MAP2 and NIBP immunofluorescent staining of mouse primary cortical neurons. NIBP expression (*red*) is colocalized with the neuronal-specific marker MAP2 (*green*).

positive. Sequence analysis of the positive clones revealed the presence of several known gene products encoding for proteins previously reported as NIK interacting partners (*e.g.* TRAF3 (36) and TRAF2 (37)). In addition, several novel clones were identified, one of which, TNAP, is a negative regulator of cytokine-induced NF-κB activation (26). In the present study we report the identification of a 211-amino acid C-terminal portion of a novel protein, NIBP. Data base analysis showed that NIBP is identical to GenBank sequence KIAA1882 (*TULARIC GENE 1*), the function of which had not been described. Human *NIBP*, a 1246-amino acid peptide (139.4 kDa), is found on chromosome 8q24.3, contains 23 exons, and shares a high degree of homology (80–90%) with rat and mouse sequences. Northern blot analysis of human tissues indicated that *NIBP* is expressed at high levels in muscle and kidney, and at lower levels in brain, heart, and placenta (Fig. 1*a*). At least two different transcripts were detected in muscle, kidney, liver, and heart, suggesting that two or more isoforms of *NIBP* may exist. Interestingly, *NIBP* transcripts were absent or only weakly detectable in immune organs and cells such as thymus, spleen, and peripheral blood leukocytes, where the NF-κB signaling pathway is known to play key regulatory roles. Immunostaining of mouse brain and spinal cord sections with an antibody raised against a peptide sequence conserved between mouse, rat, and human *NIBP* indicated the presence of NIBP in neuronal cells. Strong NIBP staining was observed in both the cell bodies and processes of neurons of the pyramidal layer of the cortex (Fig. 1*b*, *A* and *B*), in spinal cord motor neurons (Fig. 1*b*, *C* and *D*), and white matter neurons (data not shown). Positive NIBP immunolabeling was also detected in primary neurons from mouse cortex and colocalized with the neuronal-specific marker MAP2 (Fig. 1*c*).

NIBP Interacts with NIK and IKKβ and but Not IKKα or IKKγ—To confirm the interaction between NIBP and NIK in a mammalian system, Myc-tagged NIK and FLAG-tagged NIBP C-terminal 211 amino acid domains (NIBP(cd)) were coexpressed in HEK293T cells. As shown in Fig. 2*a*, NIBP(cd) was coimmunoprecipitated with NIK, indicating that they are interacting partners in the same signaling complex. Because NIK interacts with and activates the IKK complex (37, 38), we tested the hypothesis that NIBP could also interact with subunits of the IKK complex. Indeed, immunoprecipitation analysis of HEK293T cells cotransfected with Myc-NIBP and FLAG-IKKα, FLAG-IKKβ, or FLAG-IKKγ showed that NIBP interacts strongly with IKKβ, weakly with IKKα, and does not interact with IKKγ (Fig. 2*b*). The strong interaction between IKKβ and NIBP could also be detected endogenously in mouse brain lysate following immunoprecipitation with the rabbit polyclonal anti-NIBP antibody (Fig. 2*c*). Likewise, NIK was also found to interact with NIBP (Fig. 2*c*), supporting the results obtained in the overexpression system (Fig. 2*b*). No interaction was detected with either IKKα or IKKγ (Fig. 2*c*), suggesting that the weak interaction detected between NIBP and IKKα in HEK293T cells (Fig. 2*b*) could be an artifact of the overexpression system. It is noteworthy that we could detect endogenous NIK in brain lysates only following immunoprecipitation with NIBP (Fig. 2*c*). Endogenous NIBP was also detected following reciprocal immunoprecipitation of mouse brain lysate with an anti-IKKα/β and two anti-NIK antibodies (Fig. 2*d*). We ruled out that the interaction between NIBP and IKKα/β could be because of an interaction solely with IKKα, because no NIBP was detected when brain lysate was immunoprecipitated with a specific anti-IKKα antibody (Fig. 2*d*). In support of the physiological relevance of such interactions, these findings were replicated in other systems, such as in PC12 cells (Fig. 6*a*) and MCF7 cells (data not shown). By using the GST pull-down assay we determined that NIBP binding to NIK and IKKβ is direct. Interestingly, it appears that the interaction between NIK and NIBP is enhanced in the presence of IKKβ (Fig. 2*e*). Finally, no binding was detected with either IKKα or IKKγ, in agreement with our previous results in endogenous mouse brain (Fig. 2*e*).

NIBP Potentiates Cytokine-induced NF-κB Activation—To investigate the specific function of NIBP within the NF-κB cascade, we tested the hypothesis that NIBP regulates cytokine-induced NF-κB activation by its interaction with IKKβ, a key intermediate in this pathway (39).

TNFα-induced NF-κB activation was assayed in HEK293T cells overexpressing NIBP and a specific *NF-κB*-luciferase reporter construct. NIBP overexpression in untreated cells did not affect basal levels of NF-κB activation. However, in cells treated with increasing concentrations of TNFα (0.1–100 ng/ml), NIBP potentiated NF-κB activation. Similar results were obtained following treatment with IL-1β (data not shown). NIBP did not affect IFNγ-induced *interferon response factor-1*-dependent gene expression, suggesting that the potentiation of

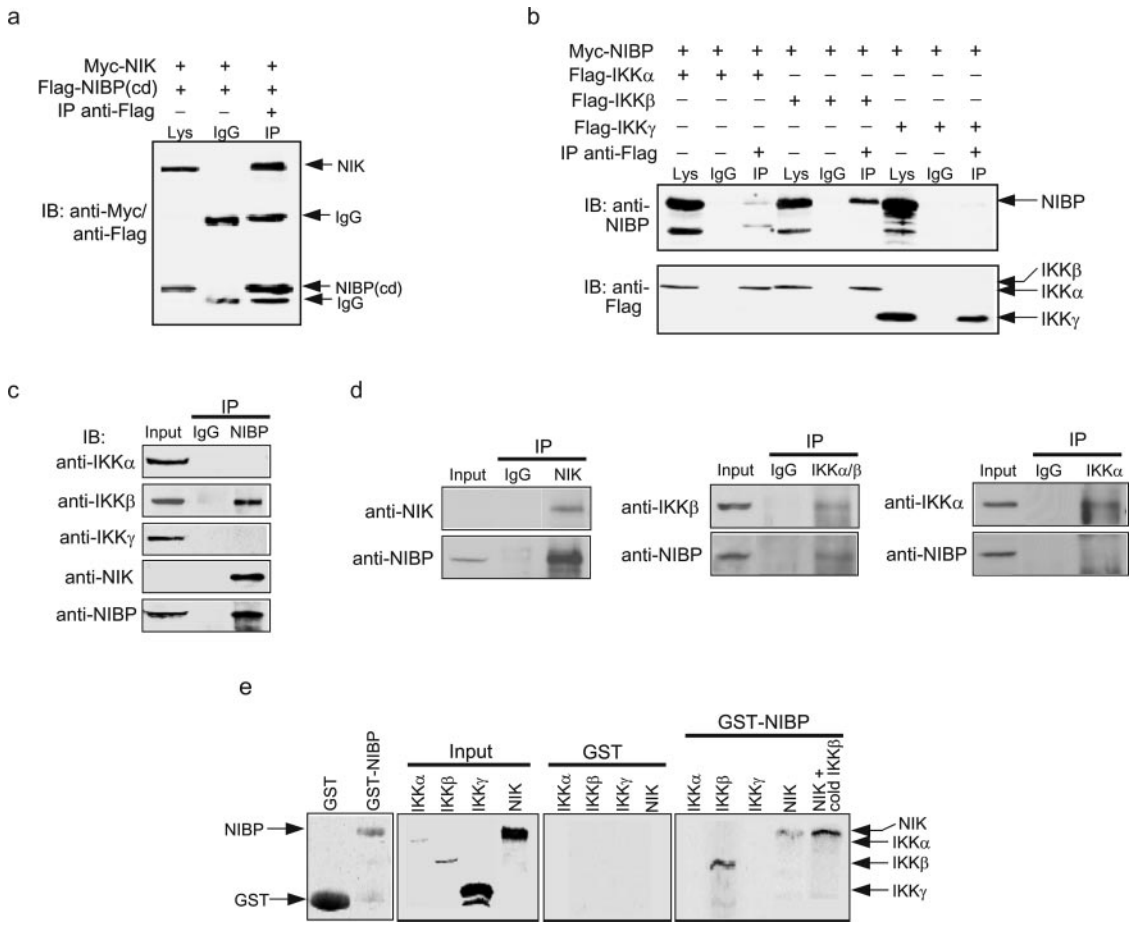

FIG. 2. **NIBP interacts with NIK and IKKβ.** *a*, lysates (*Lys*) from HEK293T cells cotransfected with Myc-*NIK* and FLAG-*NIBP*(cd) were immunoprecipitated (*IP*) with an anti-FLAG antibody and analyzed by Western blot with anti-FLAG and anti-Myc antibodies. NIK was immunoprecipitated in the presence of anti-FLAG (*IP lane*), but not in the presence of a control IgG (IgG lane). *b*, lysates from HEK293T cells cotransfected with Myc-*NIBP* and FLAG-*IKKα*, FLAG-*IKKβ*, or FLAG-*IKKγ* were immunoprecipitated with anti-FLAG and probed with an anti-NIBP antibody. NIBP was coimmunoprecipitated with IKKβ and IKKα, but not IKKγ. *c*, brain lysates were immunoprecipitated with anti-NIBP and analyzed by Western blot with anti-IKKα, anti-IKKβ, anti-IKKγ, anti-NIK, or anti-NIBP antibodies. Two different anti-NIK antibodies were used to confirm the identification of endogenous NIK. One representative blot (*IB*) is shown. IKKβ and NIK were coimmunoprecipitated with NIBP. *d*, mouse brain lysate was immunoprecipitated with anti-NIK, anti-IKKα/β, and anti-IKKα antibodies, and analyzed by Western blot with anti-NIBP, anti-NIK, anti-IKKβ, or anti-IKKα antibodies. NIBP was coimmunoprecipitated with NIK and IKKβ. *e*, GST-pull down assay to evaluate direct protein-protein interactions. Purified GST or GST-NIBP(cd) fusion protein (Coomassie staining, *left*) was incubated with *in vitro* translated [^{35}S]methionine-labeled full-length NIK, IKKα, IKKγ, or truncated IKKβ and analyzed by autoradiography. GST-NIBP interacted directly with IKKβ and NIK, and when cold IKKβ was added to ^{35}S-labeled NIK, the interaction of NIBP with NIK was enhanced (*right panel*).

gene expression by NIBP is specific to the NF-κB pathway (Fig. 3*b*).

The ability of NIBP to enhance TNFα-induced NF-κB activation was further assessed by electrophoretic mobility shift assays. Following TNFα treatment, NF-κB DNA binding activity was markedly increased in the presence of NIBP compared with control, with a maximum peak at 15 and 30 min (Fig. 3*c*). This correlated with an increased degradation of the inhibitor of NF-κB, IκBα, at the same time points of TNFα treatment (Fig. 3*d*). Taken together, these data demonstrated that overexpression of NIBP enhances cytokine-induced NF-κB activation.

To determine whether endogenous NIBP modulates NF-κB activation, we knocked down NIBP expression using a lentiviral vector-mediated siRNA approach (Fig. 4). We generated three siRNA constructs directed against the N-terminal (NR), middle (MR), and C-terminal (CR) regions of *NIBP*. To assess the efficacy and specificity of the siRNA constructs, lentiviral vector-siRNA-transduced HEK293T cells were transiently transfected with FLAG-NIBP, and ectopic and endogenous NIBP expression were measured (Fig. 4*d*). Among the siRNA constructs tested, NR markedly reduced and CR completely abolished both ectopic and endogenous expression of NIBP in HEK293T cells (Fig. 4*d*). Based on this result, we used the CR siRNA construct in subsequent knockdown experiments.

Knockdown of endogenous NIBP significantly reduced TNFα-induced *NF-κB*-dependent luciferase reporter gene expression (Fig. 4*e*). Because TNFα induces NF-κB activation through the TRAF2-NIK-IKK pathway, we tested which steps of this pathway were the targets of NIBP by overexpressing these proteins in HEK293T cells where NIBP expression was abolished by siRNA (Fig. 4*f*). Knockdown of endogenous NIBP reduced IKKβ- and NIK-mediated NF-κB activation (Fig. 4*f*), confirming that NIBP affects the function of NIK and IKKβ. As expected, because IKKβ is downstream of TRAF2, TRAF2-mediated NF-κB activation was also reduced. Because overexpression of IKKα did not markedly stimulate NF-κB activation, we cannot conclude whether NIBP affects its function (Fig. 4*f*).

To demonstrate that NIBP affects the function of NIK and the IKK complex, we examined the level of phosphorylation of IκBα and p65, which are key downstream targets of NIK-IKK in the cytokine-induced NF-κB activation pathway (40, 41). Overexpression of NIBP enhanced TNFα and IL-1β-induced phosphorylation of IκBα and p65 (Fig. 5*a*, *left*). In addition, we

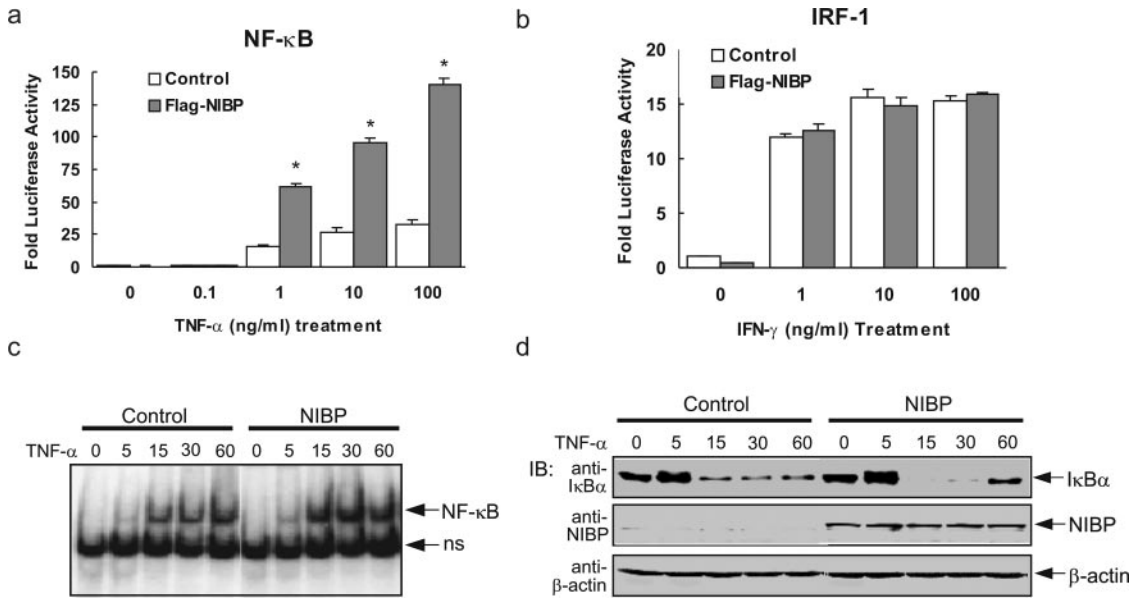

FIG. 3. **NIBP enhances TNFα-induced NF-κB activation.** *a* and *b*, HEK293T cells were cotransfected with FLAG-*NIBP* or empty vector (*control*), *NF-κB*-luciferase or *interferon response factor-1*-luciferase reporter vectors, and a *β-galactosidase* vector. Cells were treated with increasing concentrations of TNFα or IFNγ for 6 h, and luciferase and β-galactosidase activity (for normalization) were assessed. Data are expressed as relative luciferase activity compared with control, and represent the mean ± S.E. of four independent experiments run in triplicate. *, $p < 0.05$, one-way analysis of variance and Tukey test. *c*, HEK293T cells were transfected with FLAG-*NIBP* or empty vector, and NF-κB DNA binding activity was measured by electrophoretic mobility shift assay following treatment with TNFα (10 ng/ml) for the indicated time periods (minutes). *ns*, nonspecific binding. *d*, Kinetics of IκBα degradation in HEK293T cells treated with TNFα (10 ng/ml, 0–60 min), in the absence or presence of overexpressed NIBP.

evaluated IκBα and p65 phosphorylation following overexpression of IKKα, IKKβ, or IKKα/β. IKKα overexpression did not induce IκBα phosphorylation in the presence or absence of overexpressed NIBP (Fig. 5*a*, *right*), in agreement with the luciferase reporter gene assay data (Fig. 4*f*). In contrast, overexpression of IKKβ markedly induced IκBα phosphorylation, which was further potentiated in the presence of overexpressed NIBP (Fig. 5*a*, *right*). This is in agreement with our previous results showing that the absence of NIBP strongly reduces IKKβ-induced NF-κB activation (Fig. 4*f*). When coexpressed, IKKα and IKKβ activated IκBα phosphorylation (Fig. 5*a*, *right*). This was enhanced in the presence of NIBP, although to a lesser extent than with IKKβ alone. This could be because of an inhibitory effect of IKKα on IKKβ kinase activity (42). IKKα overexpression stimulated p65 phosphorylation only when coexpressed with NIBP (Fig. 5*a*, *right*). In this case, NIBP could be recruiting IKKβ and/or NIK, which would be responsible for p65 phosphorylation (40, 43). As previously reported (43), overexpression of IKKβ alone induced p65 phosphorylation (Fig. 5*a*, *right*). This was significantly enhanced by coexpression of NIBP, suggesting that NIBP may play a role in modulating IKKβ kinase activity. When coexpressed, IKKα and IKKβ induced a much higher degree of p65 phosphorylation than when expressed singularly, indicating a possible synergistic cooperation between the two kinases. We were unable to detect any further increase in phosphorylation of p65 in the presence of NIBP, possibly because of a saturation of the system (Fig. 5*a*, *right*). Because it has been reported that IKK kinase activity is dependent on the phosphorylation of the activation loop of IKKα and IKKβ (44, 45), we evaluated whether this was the mechanism of NIBP-mediated potentiation of IKK kinase activity. We measured IKKα and IKKβ phosphorylation with a phospho-specific antibody recognizing the activation loop of these kinases in HEK293T cells overexpressing IKKα, IKKβ, or IKKα/β (Fig. 5*b*). IKKα overexpression did not induce IKKα phosphorylation, whereas IKKβ overexpression stimulated IKKβ phosphorylation (45) (Fig. 5*b*). When IKKα and IKKβ were coexpressed, an increase in phosphorylation of both IKKα and IKKβ was observed (42) (Fig. 5*b*). Coexpression of IKKα with NIBP had no effect on the phosphorylation state of IKKα (Fig. 5*b*, *right*). However, IKKα and IKKβ displayed an increased phosphorylation when coexpressed with NIBP (Fig. 5*b*, *right*). We next assessed the effect of NIBP on the phosphorylation of endogenous IKKα and IKKβ. In the absence of TNFα stimulation, NIBP increased the phosphorylation of IKKα and IKKβ (Fig. 5*c*). HEK293 cells treated with TNFα exhibited a time-dependent increase in IKKα and IKKβ phosphorylation that was potentiated in the presence of overexpressed NIBP (Fig. 5*c*). Taken together, these data suggest that NIBP may be recruiting kinases such as NIK to the IKK complex, and therefore act like an adaptor protein.

Interestingly, our immunohistochemical studies in the central nervous system indicated that NIBP is predominantly expressed in neurons (Fig. 1*b*), suggesting that NIBP might perform a unique modulatory function in the neuronal NF-κB signaling pathway. To address this question, we performed a series of experiments in the PC12 neuronal cell line that endogenously expresses NIBP (Fig. 6, *a* and *b*). As previously demonstrated in HEK293T cells and brain extracts (Fig. 2), NIBP interacts with both IKKβ and NIK in PC12 cells (Fig. 6*a*). Based on recent studies demonstrating the involvement of NIK and NF-κB in NGF-induced PC12 survival and neurite outgrowth (25, 35, 46, 47), we evaluated NIBP function in NGF-induced PC12 gene expression and differentiation. After verifying that the NIBP(CR) siRNA construct effectively abolished NIBP protein expression (Fig. 6*b*), we assessed the expression of *Bcl-xL*, a survival gene induced by NGF and known to be regulated by NF-κB in PC12 cells. As predicted, knockdown of NIBP dramatically reduced NGF-stimulated *Bcl-xL* gene expression, which was restored following overexpression of NIBP (Fig. 6*c*). In unstimulated cells, overexpression of NIBP markedly up-regulated *Bcl-xL*, mimicking the effect of NGF treatment (Fig. 6*c*). These data suggest that NIBP functions as a downstream component of the NGF pathway in PC12 cells.

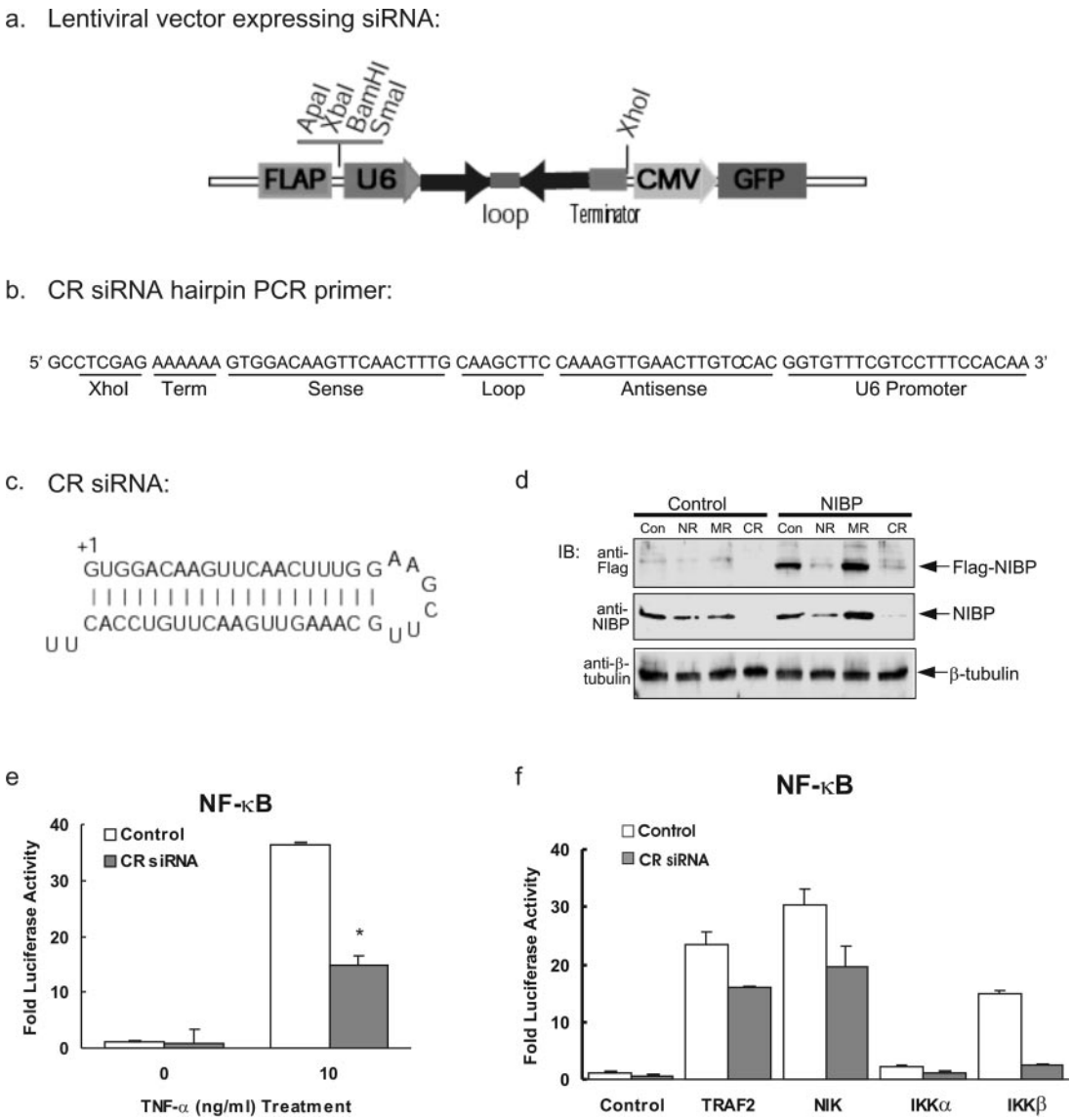

FIG. 4. **Knockdown of NIBP reduces TNFα-induced NF-κB activation.** *a*, map of the pLL3.7 lentiviral vector expressing *NIBP* siRNA. *b*, primers for cloning of hairpin *NIBP* cassette (CR). *c*, schematic representation of CR hairpin siRNA. *d*, HEK923T cells were transduced with empty (control) or lentiviral siRNA vectors targeting the N-terminal (NR), middle (MR), and C-terminal (CR) regions of mouse NIBP. After 4 weeks (infection efficiency over 98%), cells were transfected with FLAG-*NIBP* or empty vector, and analyzed by Western blot with anti-FLAG, anti-NIBP, or anti-β-tubulin (normalization control) antibodies. The NR and CR siRNA constructs efficiently knocked down NIBP expression. *e*, CR siRNA infected HEK293T cells were transfected with *NF-κB* luciferase and *β-galactosidase* reporter vectors 24 h prior to TNFα treatment (10 ng/ml, 6 h). Knockdown of endogenous NIBP significantly reduced TNFα-stimulated NF-κB-dependent luciferase activity (*, $p < 0.05$, one-way analysis of variance and Tukey test). *f*, CR siRNA-infected HEK293T cells were transfected with empty vector (control), *TRAF2*, *NIK*, *IKKα*, or *IKKβ*, and *NF-κB*-dependent gene expression was measured by luciferase reporter gene assay.

Furthermore, knockdown of endogenous NIBP with the NIBP(CR) siRNA construct prevented NGF-induced neurite extension (Fig. 6*d*). The NIBP(NR) construct, which reduces but does not completely abolish endogenous NIBP expression (Fig. 4*d*), also efficiently reduced PC12 differentiation (Fig. 6*d*). Conversely, the NIBP(MR) construct, which did not suppress NIBP expression (Fig. 4*d*), had no effect on NGF-induced PC12 differentiation (Fig. 6*d*).

DISCUSSION

Although NF-κB was initially discovered and characterized as a transcription factor required for B-cell-specific gene expression, further studies demonstrated that it is ubiquitously expressed and serves as a regulator of the expression of a wide variety of genes in many organs and tissues. In recent years, a large body of evidence has implicated NF-κB in the regulation of both physiological and pathological processes in the central nervous system (48–50). Nevertheless, little is known about the specific regulatory mechanisms of NF-κB function in this system. To address this issue, we used a yeast two-hybrid approach and identified NIBP, a novel NF-κB regulatory protein, which directly interacts with NIK and IKKβ and is required for cytokine-induced NF-κB activation.

Coimmunoprecipitation experiments with both overexpressed and endogenously expressed NIBP demonstrated the interaction of NIBP not only with NIK, originally chosen as bait in the yeast two-hybrid screening, but also with IKKβ, one of the members of the IKK complex. No interaction was detected with IKKα or IKKγ. These results point at a role of NIBP in the regulation of the classical NF-κB pathway, in which, upon cytokine stimulation, IKKβ is activated and phosphorylates IκB proteins releasing NF-κB dimers to the nucleus (51). To further corroborate this function, we demonstrated that

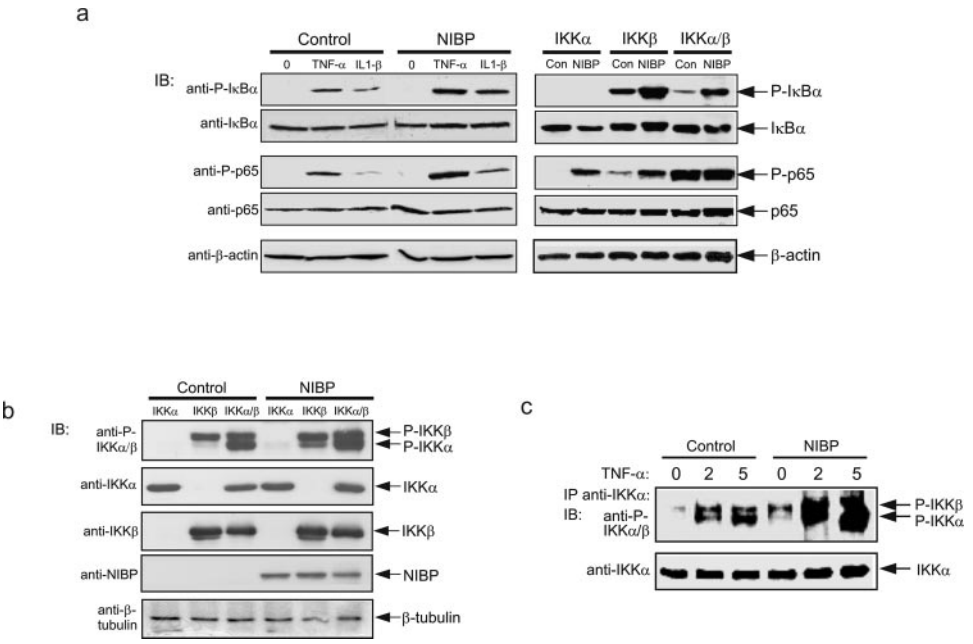

FIG. 5. **NIBP enhances cytokine-induced phosphorylation of IκBα and p65.** *a*, *left panel*, HEK293T cells transfected with *NIBP* or empty vector (control) and treated with TNFα (10 ng/ml TNFα, 10 min) or IL-1β (100 ng/ml, 10 min). *Right panel*, HEK293T cells transfected with IKKα, IKKβ, or *IKKα/β* in the presence or absence of overexpressed NIBP. In both experiments, phosphorylation levels of IκBα and p65 were evaluated by Western blot with specific anti-phospho-IκBα (Ser-32/36) and anti-phospho-p65 (Ser-536) antibodies. As controls, the same blots were reprobed for β-actin and total IκBα and p65. *b*, HEK293T cells were cotransfected with NIBP and *IKKα*, *IKKβ*, or *IKKα/β*, and analyzed by Western blot with an anti-phospho-IKKα/β (Ser-176/180) specific antibody. *c*, HEK293T cells transfected with *NIBP* or empty vector (*Con*) and either untreated or treated with TNFα (10 ng/ml, 2 or 5 min). Following immunoprecipitation (*IP*) with an anti-IKKα antibody, lysates were analyzed by Western blot (*IB*) with an anti-phospho-IKKα/β antibody. As a control, the same blots were probed for anti-IKKα.

NIBP is necessary for TNFα-induced NF-κB activation, which requires the recruitment of the classical NF-κB signaling cascade (7). Indeed, when NIBP expression is abolished, TNFα-induced NF-κB-dependent gene expression is reduced, as is the phosphorylation level of IκBα and p65, two downstream targets of IKKβ. This suggests that NIBP modulates the function of IKKβ by affecting its kinase activity. Interestingly, the interaction of NIBP with NIK is enhanced in the presence of IKKβ, suggesting that these three proteins are likely components of the same multimeric signalsome. This leads us to speculate that NIBP may act as a scaffolding protein and possibly interact with other members of the NF-κB signaling cascade that we have not yet investigated. NIBP shares some similar functions with several other scaffolding molecules of the IKK complex that have been previously identified (*e.g.* IKAP (52), ELKS (53), and TRUSS (54)). In contrast to IKAP, ELKS, and TRUSS, however, NIBP does not interact with IKKα and IKKγ. This may indicate that the role of NIBP as a scaffold is even more specialized, and therefore that NIBP is recruited to the NF-κB signaling cascade only following certain stimuli in specific cell types.

Even though NIBP does not appear to interact with IKKα or to affect its kinase activity, we cannot discard the possibility of a role of NIBP in the regulation of the alternative pathway. To specifically address this issue, we are currently evaluating NIBP function following stimulation with known activators of the alternative pathway (*e.g.* B cell-activating factor and lymphotoxin-β).

Interestingly, NIBP is detectable only at low levels in immune organs, in which NF-κB is known to perform important biological functions. On the other hand, it is highly expressed in non-immune organs such as muscle, kidney, heart, and brain. It is relevant to point out that NIBP expression in the central nervous system appears to be restricted to neurons, suggesting a highly specialized function for this protein in the modulation of NF-κB signaling in this cell type. Indeed, to adapt a ubiquitous pathway, such as the NF-κB pathway, to specific cell requirements, additional molecules like adaptors, scaffolds, activators, and inhibitors, specifically expressed in certain cell types, are necessary. Based on our results, we hypothesize that NIBP could represent one such molecule, selectively placed along the neuronal NF-κB signaling cascade to confer specificity to the activation of NF-κB in the central nervous system. That NIBP is necessary for NGF-induced neurite extension in PC12 cells further supports this hypothesis. The absence of NIBP completely prevents the ability of PC12 cells to differentiate when exposed to NGF, indicating that NIBP is an essential component of the signaling machinery required for this specific function. Our results are in agreement with earlier studies by Foehr and colleagues (25), demonstrating that PC12 differentiation is dependent on NF-κB signaling, and specifically on NIK activation, which in turn leads to downstream phosphorylation and activation of the ERK1/ERK2 MAPK pathway. Based on this evidence we can speculate that NIBP participates in this mechanism by interacting with NIK and IKKβ and possibly by activating the ERK1/ERK2 MAPK pathway.

To further confirm the critical role of NIBP in PC12 cells, we evaluated its ability to modulate the expression of the anti-apoptotic gene *Bcl-xL*. *Bcl-xL* is required for the survival of many peripheral and central neurons during development and its induction in PC12 cells following NGF treatment is dependent upon NF-κB activation (35). By both overexpression and knockdown experiments we demonstrated that NIBP is required for NGF-induced *Bcl-xL* gene expression in PC12 cells. This indicates an essential role of NIBP within the NF-κB pathway not only in the differentiation but also the survival of these cells, leading us to speculate that similar functions may be performed by NIBP *in vivo* in different neuronal populations. Ultimately, we will be able to fully appreciate these functions in an NIBP-deficient mouse, which is currently being generated in our laboratory. Taking advantage of such a model,

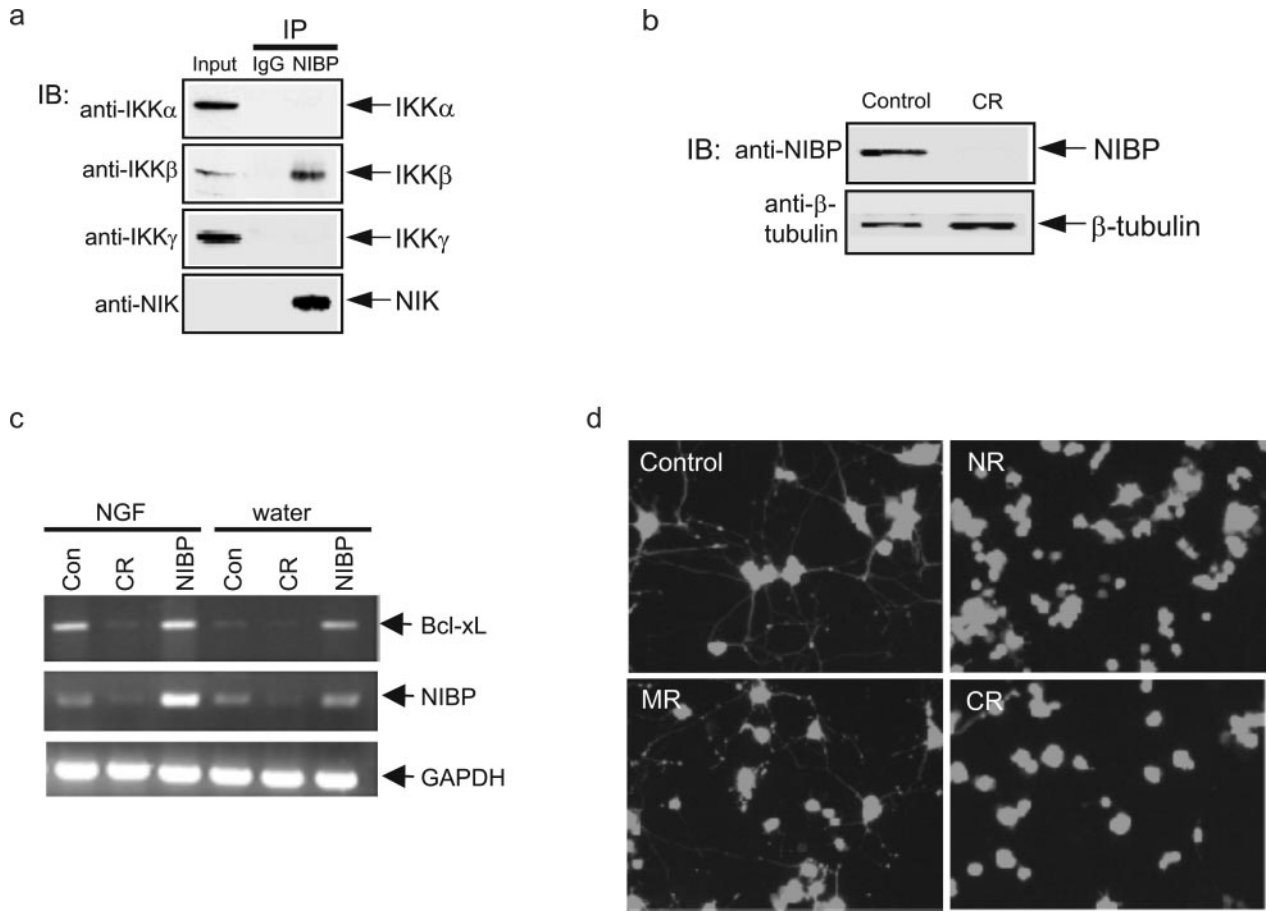

FIG. 6. **NIBP regulates NGF-induced gene expression and differentiation in PC12 cells.** *a*, PC12 cell lysates were immunoprecipitated with an anti-NIBP antibody or control IgG, and analyzed by Western blot with anti-IKK*α*, anti-IKK*β*, anti-IKK*γ*, and anti-NIK antibodies. NIBP interacted with IKK*β* and NIK, but not with IKK*α* or IKK*γ*. *b*, PC12 cells were transduced with CR siRNA or empty vector and analyzed by Western blot with anti-NIBP. *c*, *Bcl-xL* gene expression was assessed by reverse transcriptase-PCR in PC12 cells in the presence or absence of NGF stimulation. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as a control. *d*, control (*Con*) lentivirus, NR, MR, and CR siRNA-infected PC12 cells were treated with NGF (100 ng/ml). Three days following treatment, neurite extension was evaluated by fluorescent microscopy in lentivirus-transduced cells expressing enhanced green fluorescent protein.

we hope to better understand NF-κB regulatory mechanisms in neurons. This knowledge could be valuable from a therapeutic viewpoint, as NF-κB activity has been implicated in the pathophysiology of various neurodegenerative disorders.

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