IκBη, a nuclear IκB protein, positively regulates the NF-κB–mediated expression of proinflammatory cytokines

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NF-κB is a key mediator for inducible transcription of various proinflammatory genes in innate immune responses, and its activity is strictly regulated by several IκB proteins. Although signaling pathways leading from pattern recognition receptors to NF-κB's activation in the cytoplasm have been studied extensively, the detail regulatory mechanisms of NF-κB–mediated transcriptional activity in the nucleus still remain unclear. Here we describe a unique member of the nuclear IκB protein family, IκBη. In a gene expression analysis of dendritic cells, we found a unique gene encoding an uncharacterized protein with ankyrin repeats. As it was structurally related to the IκB family, the protein was named "IκBη" and further characterized in the innate immune response. IκBη was widely expressed in various tissues and predominantly located in the nucleus. Moreover, biochemical analysis showed that IκBη associated with the p50 subunit of NF-κB. Knockdown of IκBη by siRNA suppressed the transcription of a subset of NF-κB–mediated proinflammatory cytokines in LPS-stimulated and poly (I:C)-transfected macrophages. These results indicate that IκBη regulates the NF-κB–mediated transcription of a wide variety of proinflammatory genes, playing a crucial role in the regulation of innate immune responses.

innate immunity | Toll-like receptor | macrophage | signal transduction | ankyrin repeat

The NF-κB/Rel family, consisting of p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RELB, and REL (c-Rel), play a central role in the regulation of inducible gene expression in various biological systems (1–3). In response to stimuli, a dimer of NF-κB proteins binds to a κB site in the promoter or enhancer of a target gene, resulting in the expression of various inflammatory genes. The signaling pathways of NF-κB have been characterized extensively in innate immune responses. Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognize microbial components or viral nucleic acids and activate the NF-κB signal-transduction pathway to induce expression of a wide variety of inflammatory gene products, such as TNF-α, IL-6, and IFNs, playing a key role in innate immune responses (4).

NF-κB proteins are constitutively expressed in unstimulated cells, but their activities are strictly repressed by an inhibitor of NF-κB (IκB) protein (1, 5). Seven IκB proteins have been identified to date: IκBα, IκBβ, IκBε, IκBγ, BCL-3, IκBNS, and IκBζ. These proteins are characterized by multiple ankyrin repeats and interaction with an NF-κB subunit. In unstimulated cells, NF-κB forms an inert complex with a canonical IκB protein, such as IκBα or IκBβ, which masks the nuclear localization signal of NF-κB and sequesters the complex away from the nucleus. To activate NF-κB's transcriptional activity, the IκB protein needs to be released from the NF-κB/IκB complex (3). Upon stimulation, TLRs activate IκB kinase via a protein cascade, leading to phosphorylation of IκB bound to NF-κB. Phosphorylated IκB proteins were degraded by the proteasome system, releasing NF-κB from the inactive complex with canonical IκB proteins. Free NF-κB translocates into the nucleus and regulates transcription of target genes by binding to κB sites in their promoters or enhancers.

A wide variety of proinflammatory genes are regulated by NF-κB, and its regulatory mechanisms are also diverse because of cooperating with multiple regulatory factors. In addition to the canonical IκB proteins acting as inhibitors of NF-κB signal transduction in the cytoplasm, there are nuclear IκB proteins, such as BCL-3, IκBNS, and IκBζ, which are present in the nucleus and thought to regulate the transcriptional activity of NF-κB (5, 6). Although less expressed under normal conditions, these nuclear IκB proteins are highly inducible in response to stimuli. BCL-3 functions as either an activator or an inhibitor of NF-κB in a context-specific manner by regulating its transcriptional activity or its stability on DNA (7, 8). IκBNS inhibits IL-6 production by associating with DNA-bound p50 homodimers, preventing the binding of NF-κB dimers to the promoters (9, 10). IκBζ induced to express by stimuli binds to p50 on IL-6 and IL-12p40 promoters, and is supposed to activate transcription by remodeling the nucleosomes in targeted regions (11, 12). Therefore, the transcriptional activity of NF-κB is regulated not only by cytoplasmic IκBs, but also by nuclear IκBs, to strictly modulate the NF-κB–mediated expression of inflammatory genes. However, factors regulating NF-κB's activity in the nucleus still remain largely unknown.

In this article, we describe a unique molecule with ankyrin repeats that was found in a gene expression analysis of bone marrow-derived dendritic cells (BMDCs). Because it is structurally related to the IκB family, we have named the protein "IκBη" and further studied its expression and functions, especially in innate immune responses. We found that IκBη is predominantly located in the nucleus and interacts with the p50 subunit of NF-κB. We also showed that IκBη modulates the NF-κB–mediated transcriptional activity for the expression of a subset of proinflammatory genes. Based on these results, we propose that IκBη is a unique nuclear IκB protein playing a crucial role for regulating the expression of proinflammatory genes in innate immune responses.

Results

IκBη Is a Unique Member of the IκB Family. To find novel molecules involved in the regulation of immune responses, a microarray analysis was performed using mRNA from mouse BMDCs. Among many genes expressed in BMDCs, we were interested in one gene encoding ankyrin repeats, because ankyrin repeats are known to be important for interaction with NF-κB, a central regulator of inflammation (13). This gene was identical to the

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Ankrd42 gene in databases, but its function had not been described. The protein encoded by the gene consists of 516 amino acid residues and has eight ankyrin repeats in the NH_2-terminal region and a coiled-coil domain in the COOH-terminal region (Fig. 1*A*). Its amino acid sequence, especially the ankyrin-repeats domain, is highly conserved in mouse, rat, and human, and closely related to that of the IκB family, well-known regulators of NF-κB signal transduction (Fig. 1*B* and Fig. S1*A*). Moreover, the protein was similar in function to IκB proteins as described below. Therefore, we have named it IκBη and further analyzed its expression and functions.

The tissue distribution of IκBη in adult mice was examined by Northern blotting (Fig. 1*B*). An ≈2.8-kb mRNA for IκBη was rather ubiquitously expressed in all of the tissues examined and highly expressed in the brain, lung, testis, and ovary. As NF-κB mediates a central signaling pathway in innate immune responses and IκB family proteins are major components of NF-κB signal transduction, we examined the expression of IκBη in antigen-presenting cells by RT-PCR assay and revealed that IκBη was expressed not only in dendritic cells but also in macrophages in the spleen (Fig. S1*B*). IκBη was also expressed in T cells and B cells (Fig. S1*B*). These results indicate that IκBη is ubiquitously expressed in various tissues and blood cells, including antigen-presenting cells.

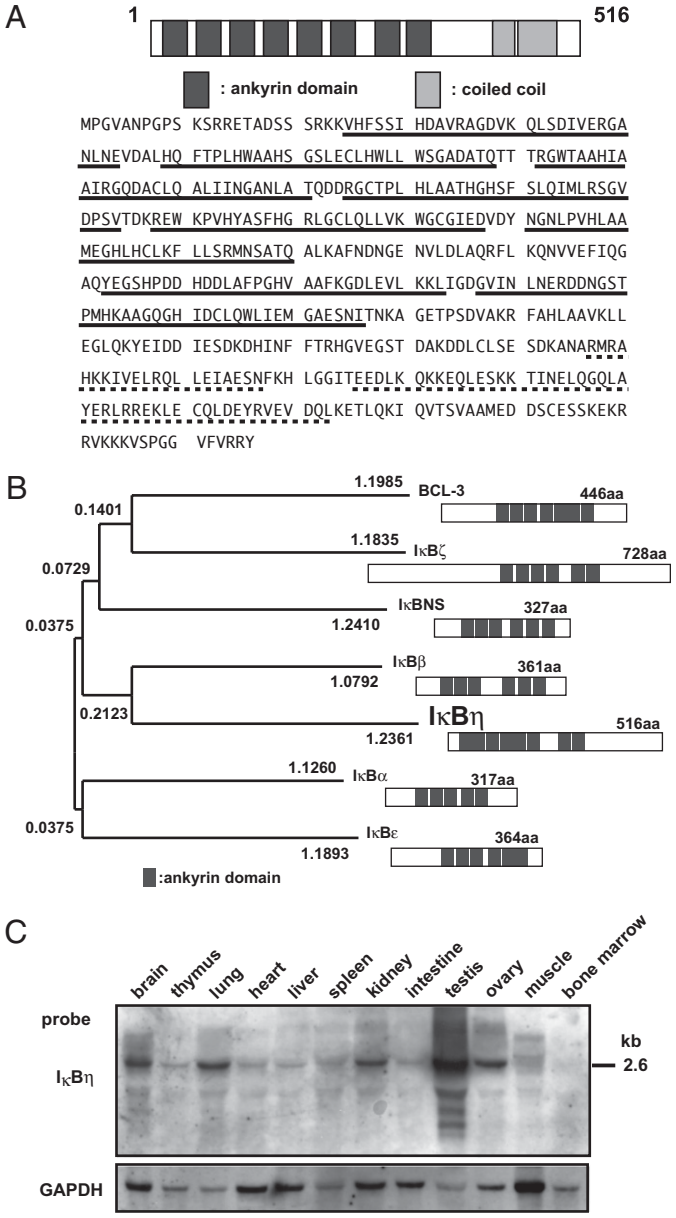

Fig. 1. Structure and tissue distribution of mouse IκBη. (*A*) The amino acid sequence and a structural diagram of IκBη. The ankyrin repeats and coiled-coil domains are indicated by blocks and boxes, respectively. Black underlines indicate ankyrin domains and the dotted underline indicates the putative coiled-coil region. (*B*) A phylogenic tree of mouse IκB family proteins, obtained using the Neighbor-Joining method. Diagrams of each IκB protein and ankyrin domains are shown on the right. (*C*) Northern blot of mouse tissues probed with mouse IκBη or the GAPDH control probe. Each lane contains 20 μg of total RNA. The position of the 2.6 kb marker is indicated.

Expression and Subcellular Distribution of IκBη. Because the expression of IκB proteins is known to be highly inducible, we examined whether the expression of IκBη was regulated by TLR signals by real-time RT-PCR (Fig. 2*A*). Expression of *Ikbh* mRNA in the macrophage cell line Raw264.7 was constitutive and only slightly up-regulated by LPS. In addition to LPS, zymosan, poly (I:C) and CpG DNA, ligands for TLR2, TLR3, and TLR9, respectively, also only marginally increased the expression of IκBη in Raw264.7 cells (Fig. 2*A*). The induction was

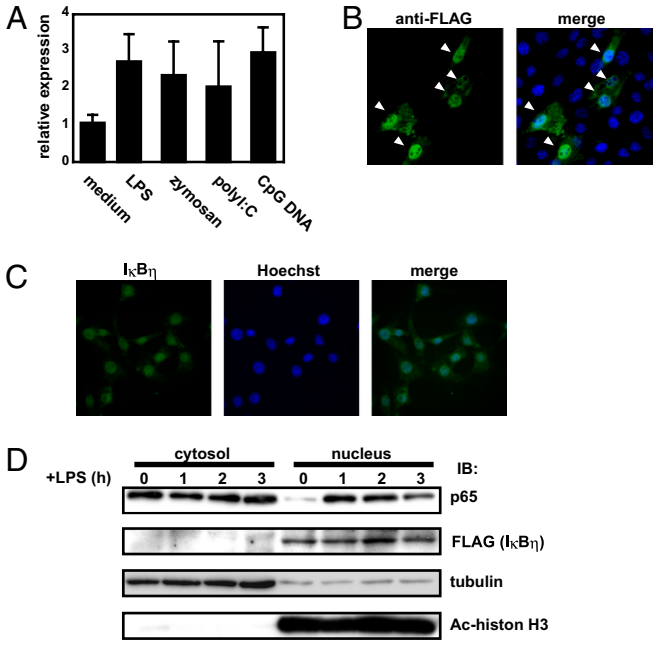

Fig. 2. Expression and subcellular distribution of IκBη. (*A*) Raw264.7 cells were treated with medium, LPS (100 ng/mL), zymosan (1 mg/mL), poly (I:C) (25 μg/mL), and CpG DNA (1 μM) for 90 min and the expression of *Ikbh* and *Hprt* mRNA was measured by real-time RT-PCR. The expression of IκBη was normalized to that of the housekeeping gene *Hprt*. The data are shown as the relative expression of *Ikbh* compared with untreated cells. (*B*) NIH 3T3 cells were transfected with FLAG-tagged IκBη for 24 h. Cells were stained with anti-FLAG Ab (green) as well as Hoechst dye (blue), and analyzed by microscopy. Arrowheads indicate transfected cells. (*C*) NIH 3T3 cells were stained with anti-IκBη Ab (green) as well as Hoechst dye (blue), and analyzed by microscopy. (*D*) Raw264.7 cells were transiently transfected with FLAG-tagged IκBη and then stimulated with LPS. Cytoplasmic and nuclear fractions were isolated and subjected to Western blotting using anti-IκBα, anti-p65, anti-FLAG Abs. Tubulin and acetyl-histone H3 were used as a cytoplasmic and nuclear marker, respectively. Data shown are representative of two or three experiments.

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much weaker compared with the induction of other nuclear IκBs (Fig. S2*A*). These results indicate that, in contrast to the mRNA of other IκBs, IκBη mRNA is constitutively expressed at a basal level and marginally up-regulated by the TLR signaling pathways in macrophages.

IκB proteins can be categorized into two groups. Canonical IκB proteins, like IκBα and IκBβ, are predominantly localized in the cytoplasm and block the nuclear translocation of NF-κB (1). On the other hand, nuclear IκB proteins, such as IκBNS and IκBζ, are present in the nucleus and regulate NF-κB's transcriptional activity (7, 9, 14). To determine the subcellular distribution of IκBη, NIH 3T3 mouse fibroblasts were transfected with cDNA encoding a FLAG-tagged IκBη, and the protein's localization was examined by immunofluorescence staining. We found that FLAG-IκBη as well as endogenous IκBη was predominantly located in the nucleus (Fig. 2 *B* and *C*). To further examine the expression and distribution of IκBη in LPS-stimulated macrophages, we next separated cytosolic and nuclear fractions of Raw264.7 cells and performed Western blotting (Fig. 2*D*). The results showed that NF-κB p65 was translocated from the cytoplasm to the nucleus in response to LPS and stayed in the nucleus for 3 h after the stimulation. However, LPS did not induce a significant change in the expression or nuclear localization of FLAG-IκBη. Taken together, these results indicate that IκBη is constitutively expressed in the nucleus regardless of the stimulation, and strongly suggest that IκBη is a nuclear IκB protein.

We constructed deletion mutants of IκBη to identify the domains required for the nuclear localization (Fig. S2*B*). FLAG-tagged full-length and deletion mutants of IκBη were expressed in NIH 3T3 cells and their distribution was examined by immunofluorescence staining (Fig. S2*C*). Full-length IκBη was present in the nucleus, but the mutants IκBη-ΔNA6 and IκBη-ΔCA2 lacking six ankyrin repeats at the N-terminal and two ankyrin repeats at the C-terminal, respectively, predominantly existed in the cytoplasm. The IκBη-ΔC mutant lacking the coiled-coil domain was present in both the nucleus and cytoplasm. These results indicate that ankyrin repeats are necessary for the nuclear localization, to which the C-terminal domain also contributes.

IκBη Interacts with the p50 Subunit of NF-κB. One key feature of IκB proteins is their interaction with NF-κB components, and nuclear IκB proteins were reported to interact with p50 or p52 rather than p65 (6). To test our hypothesis that IκBη is a unique nuclear IκB protein, we examined whether it interacts with NF-κB subunits. NIH 3T3 cells transiently expressing FLAG-IκBη were lysed and proteins immunoprecipitated with control, anti-p50, and anti-p65 antibodies were immunoblotted with anti-FLAG antibody (Fig. 3*A*). IκBη was coprecipitated with p50, but not with p65, indicating that IκBη is associated with a p50 homodimer or a heterodimer of p50 with an NF-κB subunit. Conversely, p50—but not p65—was coimmunoprecipitated with FLAG-tagged IκBη (Fig. 3*B*). We confirmed this interaction by using Myc-tagged NF-κB subunits (Fig. 3*C*). FLAG-IκBη was coimmunoprecipitated with Myc-p50, but not with Myc-p65. We also confirmed the interaction of endogenous proteins by using anti-IκBη antibody (Fig. S2*E*). These results indicate that IκBη interacts with NF-κB p50, rather than p65. It is known that ankyrin domains are important for the interaction with NF-κB (13). To determine which part is important for the association with p50, we carried out a coimmunoprecipitaton assay with IκBη deletion mutants (Fig. S2*D*). Full-length IκBη and the deletion mutant IκBη-ΔNA6 lacking the NH_2-teminal region of ankyrin repeats were expressed in NIH 3T3 cells, and the cell lysate was coimmunoprecipitated with anti-p50 antibody. Full-length IκBη, but not IκBη-ΔNA6, was found to associate with p50. These results indicated that ankyrin repeats are essential for the interaction with p50.

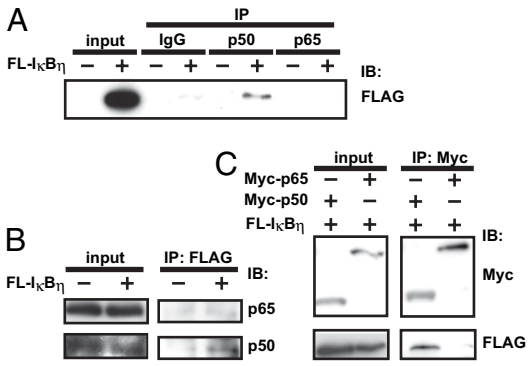

Fig. 3. Interaction of IκBη with the p50 subunit of NF-κB. (*A*) NIH 3T3 cells were transfected with control GFP or FLAG-tagged IκBη for 24 h and proteins were immunoprecipitated (IP) with anti-p50 Ab, anti-p65 Ab, or control rabbit IgG Ab. The immunoprecipitates were immunoblotted with anti-FLAG Ab. (*B*) NIH 3T3 cells were transfected as in *A* and proteins immunoprecipitated with anti-FLAG Ab. Whole-cell lysate or immunoprecipitated protein was immunoblotted with anti-p65 or anti-p50 Ab. (*C*) Cos7 cells were cotransfected with Myc-tagged p65 or p50 and FLAG-tagged IκBη for 24 h. Whole-cell lysates were immunoprecipitated with anti-Myc Ab, and immunoblotted with anti-FLAG Ab. Data shown are representative of two or three experiments.

IκBη Regulates the Expression of a Subset of Proinflammatory Genes in Innate Immune Responses. Nuclear IκB proteins are supposed to regulate the transcriptional activity of NF-κB either positively or negatively in the nucleus (7, 9, 14). To reveal the function of IκBη in innate immune responses, we first examined the effect of IκBη on transcription by NF-κB. A reporter plasmid containing three tandem repeats of the NF-κB binding site (3κB site) was transfected in NIH 3T3 cells with a control or IκBη expression vector. Expression of IκBη induced the activation of the reporter gene in NIH 3T3 cells in a dose-dependent manner (Fig. S3*A*). However, IκBη did not induce expression from the promoters in which the NF-κB binding sites were mutated. These results suggest that IκBη regulates the NF-κB signal transduction.

To confirm the function of IκBη as a regulator of NF-κB, we knocked down the expression of IκBη in Raw264.7 cells by using siRNA (Fig. 4*A*). After treatment with LPS, the production of TNF-α and IL-6 in the culture supernatant was measured by ELISA (Fig. 4 *B* and *C*). The proinflammatory cytokines were not expressed in unstimulated Raw264.7 cells and their expression was significantly up-regulated by LPS. Knockdown of IκBη in Raw264.7 cells suppressed the production of TNF-α and IL-6, although the magnitude of suppression differed. To further analyze the effects of IκBη knockdown on cytokine production in macrophages, the mRNA expression of *Tnfa*, *Il-6*, and *Ikbh* in Raw264.7 cells was quantitatively analyzed by real-time RT-PCR (Fig. S3 *B–D*). The level of *Ikbh* mRNA was reduced by IκBη siRNA regardless of LPS treatment. The expression of *Tnfa* and *Il-6* mRNA was induced by LPS stimulation, and was inhibited by IκBη siRNA. These results strongly suggest that IκBη regulates the NF-κB's transcriptional activity in the nucleus. To confirm these results, we further analyzed the mRNA expression of other genes induced at an early (*Cxcl1*, *Cxcl2*, *Il-1b*) or late (*Csf2*, *Csf3*) phase in response to LPS. Interestingly, although mRNA expression of *Cxcl2* (CXCL2) and *Il-1b* (IL-1β) was inhibited by IκBη siRNA, the expression of *Cxcl1* (CXCL1) was not significantly altered by IκBη siRNA (Fig.4 *D–F*). Similar to that of *Il-6*, the expression of *Csf2* (GM-CSF) and *Csf3* (G-CSF) mRNA was gradually induced by LPS in control cells, but was severely inhibited by IκBη siRNA (Fig.4 *G* and *H*). These results indicate that IκBη regulates the expression of a subset of NF-κB-mediated proinflammatory

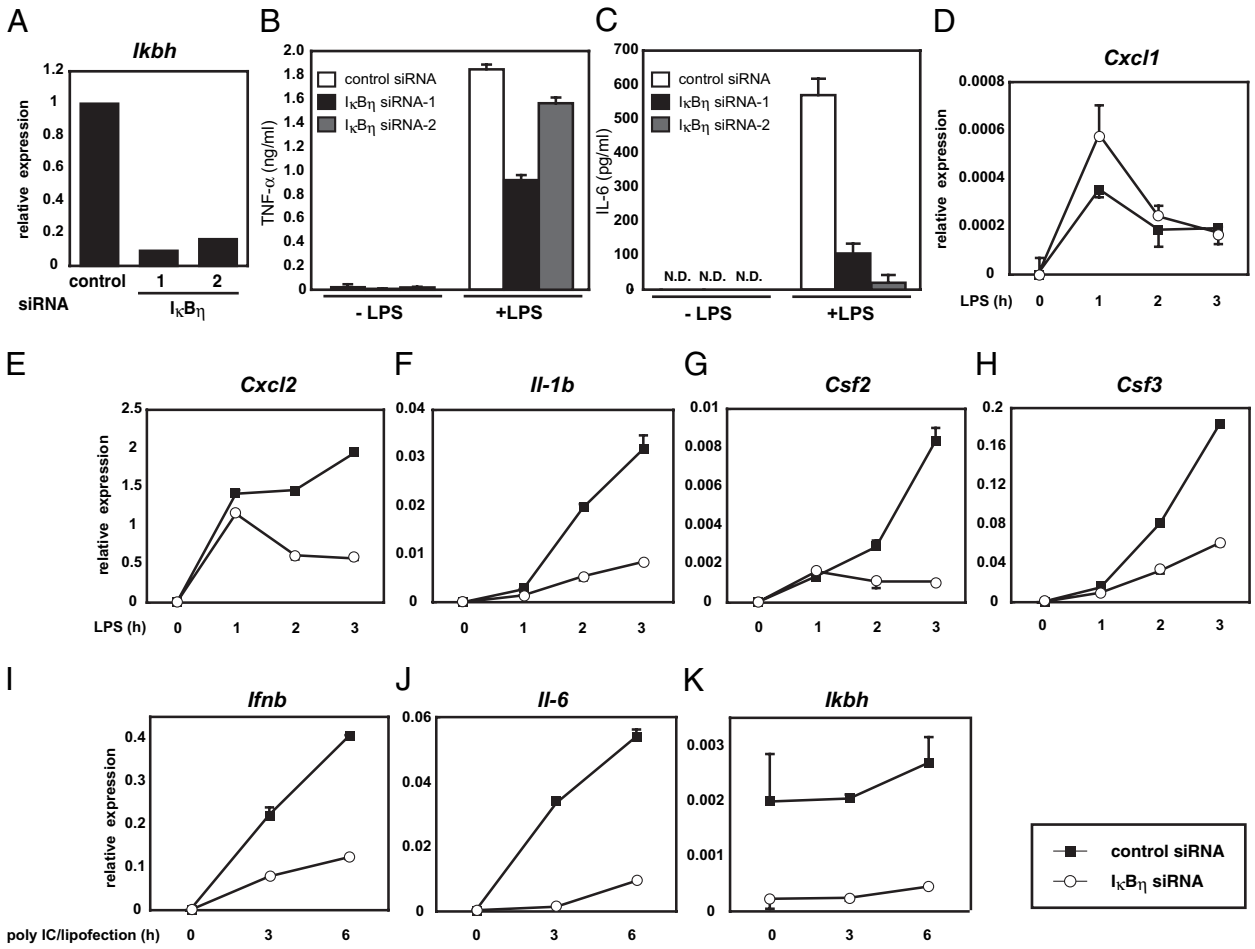

Fig. 4. IκBη regulates NF-κB-activated proinflammatory gene expressions in innate immune responses. (*A*) Raw264.7 cells were transfected with control or IκBη siRNA (1 or 2). Expression of IκBη mRNA was determined by real-time RT-PCR. The expression levels were normalized to that of the housekeeping gene *Hprt*. (*B* and *C*) Raw264.7 cells were transfected with control or IκBη siRNA, and treated with 100 ng/mL of LPS for 12 h. The levels of TNF (*B*) or IL-6 (*C*) in the culture supernatant were measured by ELISA. The data shown are representative of two experiments. (*D–H*) Raw264.7 cells were transfected with siRNA, and treated with 100 ng/mL of LPS. Total RNA was prepared at 0, 1, 2 and 3 h after the stimulation and quantitative RT-PCR was performed to measure mRNA levels of *Cxcl1* (*D*), *Cxcl2* (*E*), *Il-1b* (*F*), *Csf2* (*G*), *Csf3* (*H*). The expression levels were normalized to that of the housekeeping gene *Hprt*. Three independent experiments were performed and a representative set is shown. (*I–K*) Raw264.7 cells were transfected with control or IκBη siRNA for 26 h and then 0.5 μg/mL of poly (I:C) were transfected. Total RNA was prepared at 0, 3, and 6 h after the lipofection and mRNA expression levels of *Ifnb* (*I*), *Il-6* (*J*), and *Ikbh* (*K*) were measured by quantitative RT-PCR. The expressions were normalized to that of the housekeeping gene *Hprt*. The data shown are representative of three experiments.

cytokines. We also tested whether LPS-induced IL-10 expression affects the expression of inflammatory cytokines, because IL-10 is an anti-inflammatory cytokine induced to express by TLR stimuli and inhibits TLR-dependent gene expression (15, 16). A quantitative RT-PCR analysis revealed that IκBη knockdown did not enhance the LPS-induced *Il-10* mRNA expression in Raw264.7 cells, indicating that the suppression of LPS-induced production of proinflammatory cytokines and chemokines by IκBη siRNA was not due to the augmented expression of IL-10 (Fig. S3*E*).

It is also known that the cytosolic PRRs, such as RIG-I and MDA5, detect viral infection (17). Those RIG-I like receptors (RLRs)recognize cytosolic viral RNA and activate NF-κB to induce proinflammatory cytokines and IFNs (18). To further analyze the function of IκBη in innate immune responses, we examined the expression of *Ifnb* and *Il-6* mRNA in poly (I:C)-transfected Raw264.7 cells (Fig. 4 *I–K*). Although the expression of *Ifnb* and *Il-6* mRNA was gradually increased in poly (I:C)-transfected cells, the induction was significantly suppressed by IκBη siRNA. These results indicate that IκBη regulates the transcriptional activity of NF-κB not only in TLR signaling, but also in RLR signaling, and controls the transcription of a subset of proinflammatory cytokines in innate immune responses.

To further establish the function of IκBη, we knocked down expression of IκBη in another macrophage cell line, J774.1, and measured the production of proinflammatory genes (Fig. S3 *F–J*). IκBη siRNA significantly suppressed the expression of various proinflammatory cytokines, and the LPS-induced expression of *Il-6* mRNA in NIH 3T3 fibroblasts was also inhibited by IκBη siRNA (Fig. S3 *K* and *L*). These results indicate that IκBη plays a key role in regulating the expression of various NF-κB-mediated proinflammatory genes in various cells.

IκBη Regulates NF-κB Activity in the Nucleus. Nuclear IκB proteins are thought to modulate the activity of NF-κB at the transcriptional level, regulating the production of proinflammatory cytokines (6). To investigate the possible role of IκBη in the cytoplasmic signaling cascade, we first analyzed the effects of IκBη on LPS-induced degradation of IκBα by Western blotting (Fig. S4*A*). The IκBα level was reduced at 30 min after LPS

stimulation, regardless of the IκBη knockdown, indicating that IκBη has no effect on the stability of IκBα. We also analyzed LPS-induced phosphorylation of ERK1/2, p38, and JNK (Fig. S4*B*). Phosphorylation of these MAPKs was not altered by IκBη siRNA, indicating that knockdown of IκBη does not affect the LPS-mediated cytoplasmic signaling by MAPKs. Furthermore, we examined whether the nuclear translocation of NF-κB induced by stimuli was affected by IκBη siRNA (Fig. S4*C*). Nuclear localization of NF-κB was not affected by the knockdown of IκBη, indicating that IκBη does not regulate the nuclear translocation of NF-κB. Nuclear IκB proteins are supposed to interact with the target promoters to control transcriptional activity of NF-κB either positively or negatively (5). To address this possibility, we used an avidin-biotin-conjugated DNA-binding assay (Fig. S4*D*). Beads conjugated with the κB site of the IL-6 promoter sequence were added to lysate from Raw264.7 cells transfected with FLAG-IκBη, and the binding of p50 and p65 subunits to the IL-6 promoter sequence was analyzed by Western blotting. Although the IL-6 promoter fragment failed to pull-down p50 and p65 from unstimulated cells, both proteins were found to bind to the DNA fragment after LPS treatment. By contrast, FLAG-IκBη was found to bind to the DNA regardless of LPS stimulation. These results suggest that IκBη interacts with DNA to regulate the transcriptional activity of NF-κB.

Nuclear IκB proteins, BCL-3, IκBNS, and IκBζ, are rapidly induced by stimulation of TLRs and regulate NF-κB–mediated transcription. BCL-3 has been suggested to mainly control the transcription of primary response genes, and IκBNS and IκBζ regulate the expression of secondary response genes (10–12, 19). Because IκBη controls the expression of various proinflammatory genes, we examined the effect of IκBη siRNA on the LPS-induced expression of the three nuclear IκBs in Raw264.7 cells by real-time RT-PCR (Fig. S4 *E–G*). The siRNA did not alter the expression of these three IκBs, indicating that the inhibitory effect of IκBη siRNA on the expression of secondary response genes was not caused by the reduced expression of IκBNS and IκBζ. Similar results were also obtained by IκBη siRNA in J774.1 cells (Fig. S4*H*). Taken together, our results strongly suggest that IκBη directly regulates the expression of NF-κB-mediated transcription.

Discussion

NF-κB plays a central role in the inducible transcription of various proinflammatory genes. Prompt responses to inflammatory stimuli rely on repression of the transcriptional activity of NF-κB by inhibitors known as IκB proteins (1, 3). In the cytoplasm, canonical IκB proteins form a complex with NF-κB and inhibit its nuclear translocation (20). Extracellular and intracellular stimuli, such as TLR and RLR ligands, respectively, induce the ubiquitination and degradation of IκB, allowing NF-κB to move into the nucleus to regulate gene expression (17). In contrast to canonical IκB proteins, nuclear IκB proteins play a regulatory role in NF-κB–mediated transcription (6). In this article, we described a unique protein with eight ankyrin repeats, named IκBη, because of its structural similarity to the IκB family (Fig. 1*B*). We have also provided evidence that IκBη plays a crucial role as a nuclear IκB for regulating the NF-κB–mediated transcription of various proinflammatory genes in innate immune responses.

IκBη localizes in the nucleus and the subcellular localization is not affected by LPS treatment (Fig. 2 *B–D*). In addition, we found that IκBη binds to the p50 subunit of NF-κB via its ankyrin repeats (Fig. 3 *A–C*). Consistent with these results, it is known that nuclear IκB proteins prefer to interact with the p50 or p52 subunit of NF-κB, rather than other Rel family proteins (6). Although BCL-3, IκBNS, and IκBζ possess a nuclear localization signal motif and are predominantly located in the nucleus, IκBη lacks a canonical nuclear localization signal motif. Deletion analysis showed that the ankyrin repeats are absolutely necessary for the nuclear localization of IκBη, to which the coiled-coil domain also contributes (Fig. S2*C*). Moreover, compared with other nuclear IκB proteins whose expression is highly inducible through TLR signaling, IκBη is unusual because it is constitutively expressed in various tissues and only marginally up-regulated by TLR signaling (Fig. S2*A*).

Knockdown experiments using siRNA revealed that IκBη regulates NF-κB–mediated expression of a subset of proinflammatory genes in LPS-stimulated macrophages (Fig.4 *A–H* and Fig. S3 *D–J*). We also demonstrated that IκBη regulates the expression of *Ifnb* and *Il-6* mRNA in poly (I:C)-transfected macrophages (Fig. 4 *I–K*). These results strongly suggest that IκBη plays a crucial role for the expression of proinflammatory genes in innate immune responses. Moreover, IκBη positively regulates *Il-6* mRNA expression not only in macrophages but also in NIH 3T3 fibroblast cells, suggesting that IκBη regulates gene expression in various cell types (Fig. S3 *K* and *L*).

TLR-induced proinflammatory genes are divided into two subclasses, primary and secondary response genes (4). Primary response genes, such as *Tnfa* and *Cxcl2*, are expressed immediately in the absence of protein synthesis; the expression of secondary response genes, including *Il-6* and *Csf3*, requires newly synthesized mediators of NF-κB and occurs after the primary responses (21). IκBζ is considered to be essential for the induction of various secondary response genes, but not for primary genes, and IκBNS also regulates only secondary response genes (11, 22). In contrast to those known nuclear IκB proteins, IκBη regulates both types of genes, possibly because it is constitutively expressed in the nucleus (Fig. 4 *E–H* and Fig. S3 *B–J*).

As IκBη predominantly exists in the nucleus and it has no effect on the degradation of IκBα or nuclear translocation of NF-κBs, it is plausible that IκBη regulates NF-κB transcriptional activity in the nucleus (Fig. S4 *A–C*). In fact, we also showed that IκBη interacts with the DNA fragment of the IL-6 promoter, suggesting that IκBη regulates the transcriptional activity of NF-κB on promoters (Figs. S4*D* and S5). However, promoter activity of proinflammatory genes is strictly regulated in vivo by multiple mechanisms, such as chromatin remodeling, stable recruitment of transcriptional factors or cofactors, and posttranslational modification of NF-κB (12, 21, 23–25). Thus, it is possible that IκBη regulates chromatin remodeling as well as formation and stability of the transcriptional complex, and there may be an additional unknown mechanism for IκBη to modulate the transcriptional activity of NF-κB.

Although IκBη regulates the expression of a wide variety of proinflammatory genes, it has little or no effect on the expression of some NF-κB–regulated genes, such as *Cxcl1* and *Ikbz* (Fig. 4*D* and Fig. S4 *E–H*). It has been reported that NF-κB regulates expression of a distinct set of target genes by a distinct mechanisms, in cooperation with multiple regulatory factors, including p300/CBP cofactor or Trap80 subunit of the Mediator complex (24, 26). IκBη may also regulate a subset of NF-κB target genes on specific promoters or enhancers in a context-dependent manner. Taken together, the precise regulatory mechanism of IκBη still remains to be studied and is an interesting and important subject of further investigation.

In conclusion, IκBη is a unique nuclear IκB protein that contributes to NF-κB–mediated transcription, and plays an important regulatory role in innate immune responses by regulating the expression of proinflammatory cytokines (Fig. S5). Although this study focuses on the innate immune responses, ubiquitous expression of IκBη suggests that it may also play an important role for regulation of NF-κB signaling in other biological systems (27, 28).

Materials and Methods

Plasmids and Transfection. IκBη cDNA cloned by PCR was inserted into the vector pME-18S with a FLAG-tag, and p50 and p65 cDNA were cloned into

the vector pcDNA3.1 with a Myc-tag. The mutants IκBη-ΔNA6 (amino acids 251–516), IκBη-ΔCA2 (amino acids 1–230 plus 371–516) and IκBη-ΔC (amino acids 1–340) were constructed in pME-18S-FLAG. NIH 3T3 cells, Raw264.7 cells, and Cos7 cells were transfected using Attractene (Qiagen), according to the manufacturer's instructions.

RT-PCR and Real-Time RT-PCR. Raw264.7 cells, J774.1 cells, and NIH 3T3 cells were stimulated with 100 ng/mL of LPS or lipofected with 0.5 μg/mL of poly (I:C), and then total RNA was prepared using a High Pure RNA isolation kit (Roche Applied Science). The total RNA was reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Conventilnal PCR was performed with Blend Taq (TOYOBO). Quantitative real-time RT-PCR was performed on a LightCycler (Roche Applied Science) using SYBR Premix Ex Taq reagent (TaKaRa Bio Inc.). *Hprt* was used as an internal control. The primers used are listed in Tables S1 (RT-PCR) and S2 (real-time RT-PCR).

RNA Interference. For RNA interference experiments, synthetic siRNA (obtained as Stealth select RNA interference from Invitrogen) targeting mouse Ankrd42 (Table S3) or control siRNA in the mouse genome (Stealth RNAi-negative control, Medium GC duplex; Invitrogen) was transfected into Raw264.7 cells, J774.1 cells, or NIH 3T3 cells using HiPerFect (Qiagen) according to the manufacturers' directions. After 26 h of transfection, cells were collected and real-time PCR was conducted to examine the effect of the knockdown.

See *SI Materials and Methods* for further discussion.

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