Gene-specific Requirement of a Nuclear Protein, IkB-ζ, for Promoter Association of Inflammatory Transcription Regulators*

Received for publication, March 18, 2008; in revised form, August 25, 2008; Published, JBC Papers in Press, September 29, 2008, DOI 10.1074/jbc.M802148200

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Expression of many inflammatory genes is induced through activation of the transcription factor NF-κB. In contrast to the advanced understanding of cytoplasmic control of NF-κB activation, its regulation in the nucleus has not been fully understood despite its importance in selective gene expression. We previously identified an inducible nuclear protein, IkB-ζ, and demonstrated that this molecule is indispensable for the expression of a group of NF-κB-regulated genes. In this study, we established a unique gene induction system, in which IkB-ζ is expressed independently of inflammatory stimuli, to specifically investigate the molecular basis underlying IkB-ζ-mediated gene activation. We show that in the presence of IkB-ζ other primary response genes are dispensable for the expression of the target secondary response genes. ChIP analyses revealed that IkB-ζ is required for stimulus-induced recruitment of NF-κB onto the target promoter in a gene-specific manner. Surprisingly, IkB-ζ is also necessary for the gene-selective promoter recruitment of another inflammatory transcription factor, C/EBPβ, and the chromatin remodeling factor Brg1. We propose a new gene regulatory mechanism underlying the selective expression of inflammatory genes.

Many cellular responses are mediated by orchestrated gene expression. When cells are exposed to diverse inflammatory stimuli, such as microbial components, a large number of genes are induced to elicit inflammatory responses. These genes include cytokines/chemokines, anti-microbial peptides, and cell adhesion molecules, and many of them are known to be induced through activation of the transcription factor nuclear factor (NF)κB (1–3). In resting cells, NF-κB is sequestered in the cytoplasm by its inhibitors IkB-α, -β, and -ε. Upon receptor stimulation, dozens of receptor-associated molecules induce activation of the IkB kinase, resulting in degradation of the IkBs by the ubiquitin-proteasome pathway. NF-κB liberated from IkBs is translocated into the nucleus, where it participates in the transcriptional activation of target genes. As the cytoplasmic activation of NF-κB does not require new protein synthesis, this transcription factor is capable of inducing target inflammatory genes within a short period.

Nonetheless, the induction of NF-κB-regulated genes is not solely defined by the nuclear entry of NF-κB. Growing evidence suggests that each NF-κB-regulated gene has its own expression profile (for example, kinetics, cell type/stimulus-specificity and requirements of regulators), indicating the importance of gene-specific regulation after the nuclear translocation of NF-κB. Expression of individual inflammatory genes with their own profiles is considered to be crucial for the integrity of particular inflammatory responses. In contrast to the advanced comprehension of the regulatory machinery of NF-κB in the cytoplasm, the regulation of NF-κB in the nucleus is not fully understood (4, 5).

Recently, it was proposed that inflammatory genes could be categorized into two groups based on their requirement of de novo protein synthesis for induction (6, 7). While primary response genes are rapidly induced independently of new protein synthesis, induction of secondary response genes takes longer and is impeded in the presence of protein synthesis inhibitors. Thus, the expression of at least one primary response gene is assumed to be necessary for the induction of a set of secondary response genes.

We previously cloned an inducible nuclear protein, IkB-ζ, by screening the genes rapidly induced in response to a cell wall component of Gram-negative bacteria, lipopolysaccharide (LPS), in macrophages (8). Other two groups have reported the identical molecule through similar screening of inducible genes upon inflammatory stimulation (9, 10). Expression of IkB-ζ is barely detectable in unstimulated cells, but is robustly induced upon inflammatory stimulation. IkB-ζ harbors six ankyrin repeats, which are most similar to those of other nuclear IkB proteins, Bcl-3 and IkBNS. Similar to them (11, 12), IkB-ζ pref-

immunoprecipitation; sMT, sheep metallothionein; siRNA, small interfering RNA.
erentially binds to the NF-κB p50 subunit (8, 13). Subsequent analyses revealed that IkB-ζ is indispensable for the expression of a group of NF-κB-regulated genes (13). IkB-ζ is encoded by a primary response gene, NfkbiZ, and its induction depends on NF-κB activation (14–16), suggesting that IkB-ζ-regulated genes are induced via a two-step machinery. In fibroblast cells, IkB-ζ was induced in response to LPS and IL-1β, but not TNF-α (8). We have shown that TNF-α induced the transcription of the NfkbiZ gene but did not stabilize IkB-ζ mRNA, indicating that the stimulus-specific expression of IkB-ζ is determined post-transcriptionally (15).

It is still unknown how IkB-ζ is involved in the transcriptional activation of its target genes. First, the requirement of NF-κB for the expression of IkB-ζ itself makes it difficult to directly define whether or not NF-κB is indeed involved in IkB-ζ-mediated gene activation. In the present study, we devised a gene expression system in which IkB-ζ is induced independently of NF-κB activation. This system is composed of zinc-induced IkB-ζ expression and cellular activation by TNF-α stimulation, the latter of which activates NF-κB, but does not induce IkB-ζ expression. Using this system, we extended the characterization of IkB-ζ-mediated gene regulation and identified components that are essential for this process. Importantly, our ChIP experiments revealed the gene-specific requirement of IkB-ζ for the promoter recruitment of NF-κB and other transcription regulators involved in inflammatory responses. These findings suggest that IkB-ζ plays a critical role in the selective gene expression by controlling the association of key transcription regulators with target promoters in the nucleus.

**EXPERIMENTAL PROCEDURES**

_Cells, Mice, and Antibodies—_Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. IkB-ζ-deficient mice were previously described (13). Mouse embryonic fibroblasts (MEFs) and bone marrow-derived macrophages (BMMs) were prepared as previously described (17, 18). LPS from Escherichia coli 0111:B4, cycloheximide (CHX), and Helenalin were purchased from List Biological Laboratories (Campbell, CA), Nakalai Tesque (Kyoto, Japan) and Alexis Biochemicals (Lausen, Switzerland), respectively. Mouse recombinant IL-1β and TNF-α proteins were from Wako Pure Chemical Industries (Osaka, Japan). All of the antibodies used in this study were obtained from Santa Cruz Biotechnology, except for the anti-IkB-ζ antibody (15).

_Construction of Plasmids and Stable Cell Lines—_The DNA fragment containing the sheep metallothionein Ia (sMT-Ia) promoter (+600/+72) was obtained by PCR using sheep genomic DNA as template. The CMV promoter region in pcDNA3 (Invitrogen) was replaced with the sMT-Ia promoter using the restriction sites BglII and HindIII. The IkB-ζ fragment of coding region was inserted into the resultant vector. NIH3T3 cells were transfected using FuGENE6 according to the manufacturer’s instructions (Roche Applied Science), and cells were selected in the presence of 1 mg/ml G418 (Nakalai) to obtain stably transfected cells.

_Quantitation of mRNA Levels by Real Time PCR—_Total RNA isolated using TRIzol (BIOLINE, London, UK) was reverse-transcribed by ReverTra Ace (TOYOBO, Osaka, Japan). The cDNA was analyzed by quantitative real-time PCR (RT-qPCR) using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) on the Rotor-Gene 6200 system (Corbett Life Science, Sydney, Australia). The primer sequences used for RT-qPCR analyses are available upon request.

**Immunoblotting of Nuclear and Total Cellular Proteins—** Nuclear protein was extracted as described (6, 16). After cells were lysed with the buffer containing 10 mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 0.5% Nonidet P-40, and 1 mM dithiothreitol, on ice for 10 min, nuclei were pelleted by centrifugation at 2,500 × g for 5 min at 4 °C. Nuclear proteins were extracted with the buffer containing 10 mM HEPES (pH 8.0), 25% glycerol, 0.6 M KCl, 1.5 mM MgCl2, and 0.2 mM EDTA, on ice for 30 min and separated from debris by centrifugation at 15,000 × g for 5 min. The immunoblot was performed as described previously (8).

_ChIP (Chromatin Immunoprecipitation) Assay—_ChIP was performed as previously described (16, 19). Cells were fixed with formaldehyde (1%) at room temperature for 10 min and washed with ice-cold phosphate-buffered saline, twice. After a sonicated chromatin solution was pre-cleared with protein G-Sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) at 4 °C for 2 h, the antibody of interest (1.5 μg) was added and incubated at 4 °C overnight with continuous rotation. Antigen-antibody complexes captured by protein G-Sepharose were extensively washed, and DNA-protein complexes were eluted with elution buffer containing 1% SDS and 100 mM sodium bicarbonate. After cross-links were reversed by incubation at 65 °C overnight, proteins were digested with proteinase K (0.1 mg/ml) at 45 °C 3 h. 1/25th of the DNA preparation was used for PCR analysis.

**Luciferase Reporter Assay—** Cells were transfected with a luciferase reporter plasmid and the internal control plasmid pRL-TK (Promega). Two days after transfection, cells were stimulated, and luciferase activities were determined by the Dual-Luciferase Reporter assay system (Promega).
Stealth RNAi negative control duplexes (Invitrogen) were used as controls. Two days after transfection, cells were stimulated and subjected to RT-qPCR or immunoblotting.

RESULTS

A Gene Expression System for IκB-ζ-regulated Genes—We attempted to construct a gene induction system, in which IκB-ζ is expressed independently of NF-κB activation. Because the basal level of IκB-ζ is very low, we chose an inducible expression system to avoid undesirable artificial effects caused by its constitutive overexpression. The sheep metallothionein Ia (sMT-Ia) promoter is reportedly activated in response to zinc treatment exclusively (20, 21). The sMT-Ia promoter was activated when NIH3T3 fibroblast cells were treated with zinc, but was unresponsive to LPS stimulation (Fig. 1A). By contrast, the control ELAM1 reporter selectively responded to LPS. We generated a transgene, in which the coding region of IκB-ζ was placed downstream of the sMT-Ia promoter, and stably introduced it into NIH3T3 cells. Treatment of the transfected cells with zinc induced IκB-ζ protein with similar kinetics to the induction of endogenous IκB-ζ protein in response to IL-1β and LPS (Fig. 1, B and C, Ref. 15). As we have shown previously, TNF-α stimulation minimally induced IκB-ζ protein (Fig. 1C, Refs. 8 and 15).

The expression of Lcn2 (Lipocalin-2, 24p3), Csf3 (G-CSF), and Cebp (C/EBPδ) is highly dependent on IκB-ζ in MEFs (Fig. 2A). In the cells harboring the sMT-Ia-IκB-ζ transgene, these IκB-ζ-dependent genes were not significantly induced in response to either TNF-α or zinc alone, but co-treatment of the cells with TNF-α plus zinc elicited their robust expression (Fig. 2B, left panels). These genes were not expressed in control cells (Fig. 2B, right panels). By contrast, the primary response genes Tnfaip3 (A20), Cxcl1 (KC) (Fig. 2B), and Cxcl2 (MIP-2) (data not shown) were induced in response to TNF-α alone, and their expression was largely unaffected by the addition of zinc. The zinc-induced expression of IκB-ζ protein and TNF-α-induced nuclear translocation of p65 were confirmed by immunoblotting (Fig. 2C).

IκB-ζ Is the Only Primary Response Gene Necessary for Its Target Gene Induction—IκB-ζ-regulated genes are secondary response genes, because their expression was impaired in the

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Retroviral Infection and Small Interfering RNA (siRNA) Experiments—Gene transduction via retroviral infection was carried out as previously described (17). Supernatant of 293T cells transfected with Moloney Murine Leukemia virus-ΨA helper plasmid and pBABEpuro construct was used for the infection. Transfection of cells with siRNA was performed using Lipofectamine2000 as previously described (18) according to the manufacturer’s instructions (Invitrogen). Duplexed modified RNA oligonucleotides (Stealth RNAi) were obtained from Invitrogen. The sequences of the sense strands of the siRNAs were as follows: 5′-AGAAUGGACAGAAACAGCAG-GAUGU-3′ for the mouse NF-κB p50 subunit 1, 5′-GCAAGAC-GGCCCAUCUUCAAAUAU-3′ for the mouse NF-κB p50 subunit 2, 5′-GCGAUUCCAAUAGCUUCCGAGAAACCC-3′ for the mouse NF-κB p65 subunit 1 and 5′-UCCCAUUGUCUC-UCCGGAGAUGAA-3′ for the mouse NF-κB p65 subunit 2.


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To better understand the molecular machinery involved, we examined the requirement of individual NF-κB subunits using siRNAs (Fig. 5). While both p65 and p50 subunits were necessary for maximal induction of Lcn2, another IκB-ζ-regulated gene, Csf3, required only p50 for its induction when cells were treated with TNF-α plus zinc (Fig. 5, A and C). Induction of Tnfaip3 was impaired by p65 siRNA, but not by p50 siRNA. In contrast to the results of stimulation with TNF-α plus zinc, the induction of Csf3 in response to LPS required the p65 subunit, which could be explained by a requirement of p65 for IκB-ζ induction in response to LPS (Fig. 5E). The efficacy of siRNA-mediated gene knockdown was confirmed by immunoblotting (Fig. 5, B and D). It has been reported that Helenalin, a sesquiterpene lactone, is an alkylating agent specific for NF-κB p65 subunit while it does not modify the DNA binding of p50 (22). To validate the gene-specific requirement of p65 subunit, we examined sensitivity of the Lcn2 and Csf3 expression to Helenalin. Induction of Lcn2 in response to TNF-α plus zinc, but not that of Csf3, was specifically suppressed in the presence of Helenalin (Fig. 5F).

We have recently shown an involvement of C/EBP family proteins in addition to NF-κB in IκB-ζ-mediated activation of target promoters (18). To explore the involvement of C/EBP proteins in IκB-ζ-mediated gene induction, we tested the effect of expression of a dominant-negative isofrom of C/EBPB, LIP (Fig. 6A). As LIP was supposed to form non-functional heterodimers with other C/EBP family members (23, 24), expression of LIP seems to be effective for the functional suppression of members of this redundant protein family. The expression of LIP inhibited induction of Lcn2 and Csf3, but did not largely affect that of Tnfaip3 and Cxcl1. The expression of LIP did not affect zinc-inducible expression of IκB-ζ protein (Fig. 6B).

IκB-ζ Is Required for Promoter Recruitment of Inflammatory Transcription Regulators in a Gene-specific Manner—To elucidate the role of IκB-ζ in the nucleus, we examined the requirement of IκB-ζ for the recruitment of NF-κB onto target promoters by ChIP experiments (Fig. 7). When the cells harboring the sMT-la-IκB-ζ transgene were stimulated with either TNF-α or zinc alone, the NF-κB p65 subunit was not detectable at the Lcn2 promoter. By contrast, p65 was recruited to the Cxcl1 and Cxcl2 promoters upon TNF-α stimulation. Remarkably, co-stimulation of cells with TNF-α plus zinc induced p65 recruitment to the Lcn2 promoter with slow kinetics, suggesting a requirement of IκB-ζ expression for this recruitment process.

We also examined the promoter recruitment of C/EBPβ (Fig. 7). Unlike p65, Cxcl1 and Cxcl2 promoters were not enriched with C/EBPβ in a stimulus-dependent manner. Surprisingly,
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FIGURE 4. Essential requirement of NF-κB for IκB-ζ-mediated gene induction. NIH3T3 cells harboring sMT-la-IκB-ζ were retrovirally transduced with GFP or IκB-ζ(SR). A, cells were stimulated with TNF-α (5 ng/ml) plus ZnSO4 (50 μM) for the indicated periods, and total RNA was analyzed by RT-qPCR. mRNA expression levels were normalized to those of Rpl32 (B). Cells were untreated or treated with ZnSO4 (50 μM) for 4 h, and total cellular protein was analyzed by immunoblotting using the indicated antibodies. The results are representative of three independent experiments.

FIGURE 5. NF-κB subunits required for IκB-ζ-mediated gene induction. A–E, NIH3T3 cells harboring sMT-la-IκB-ζ were transfected with the indicated siRNAs. Two days after siRNA transfection, cells were stimulated with TNF-α (5 ng/ml) plus ZnSO4 (50 μM) for the indicated periods, and total RNA was analyzed by RT-qPCR. mRNA expression levels were normalized to those of Rpl32. B and D, cells were untreated or treated with ZnSO4 (50 μM) for 4 h and total cellular protein was analyzed by immunoblotting using the indicated antibodies. F, NIH3T3 cells harboring sMT-la-IκB-ζ were pretreated with Helenalin (1 μM) for 1 h, and then stimulated with TNF-α (5 ng/ml) plus ZnSO4 (50 μM). Total RNA was analyzed by RT-qPCR, and mRNA expression levels were normalized to those of Rpl32. The results are representative of at least three independent experiments.

C/EBPβ was recruited onto the Lcn2 promoter only when cells were stimulated with TNF-α plus zinc, indicating that the promoter recruitment of C/EBPβ is also dependent on IκB-ζ. It was recently reported that induction of secondary response genes is accompanied by remodeling of nucleosomes, and that this process is mediated by the two ATPase subunits of the SWI/SNF complex, Brg1 and Brm1 (7). Intriguingly, as with p65 and C/EBPβ, stimulus-dependent Brg1 recruitment to Lcn2 promoter also requires IκB-ζ (Fig. 7).

The requirement of IκB-ζ for the association of these transcription regulators with specific target promoters was also investigated in LPS-stimulated macrophages. The expression of Lcn2 gene was highly dependent on the presence of IκB-ζ in BMMs, and we observed a gene dose dependence (Fig. 8A). Consistent with the ChIP results obtained from the fibroblasts harboring the sMT-la-IκB-ζ transgene, p65, c-Rel, C/EBPβ, and Brg1 were all localized to the Lcn2 promoter only when IκB-ζ is present (Fig. 8B). The recruitment of these transcription regulators to the control promoters (Cxcl1 and Cxcl2) was not dependent on IκB-ζ. Taken together, these findings suggest that IκB-ζ determines the accessibility of target promoters to these transcription regulators in the nucleus.

DISCUSSION

In the present study, we established a unique expression system to specifically analyze the IκB-ζ-mediated gene regulation. This system allowed us to directly define essential components involved in the process.

Overexpression of IκB-α(SR) in our system revealed that the activation of NF-κB is not only required for IκB-ζ induction but also is substantially involved in the transcriptional up-regulation of the IκB-ζ-regulated secondary response genes. As the NF-κB p65 subunit is necessary for induction of IκB-ζ expression and IκB-ζ controls the
participation of p65 in the transcriptional up-regulation of selective target genes, these secondary response genes are not regulated by a simple two-step machinery in which distinct molecules are sequentially activated. Rather, it is likely that NF-κB and IkB-ζ comprise a reciprocally limiting loop to activate the selective genes. The physiological significance of this feedback mechanism will be discussed below. It has been reported that the stimulus-induced recruitment of p65 to the secondary response promoters is slower than that to the primary response promoters (19). This delay could be accounted for by the time necessary for IkB-ζ induction.

Using our gene expression system, we also demonstrated the direct involvement of the C/EBP protein in activation of IkB-ζ-regulated genes (Fig. 6). The ChIP experiments suggested that IkB-ζ regulates accessibility of the selective promoters to C/EBP proteins as well as NF-κB p65 (Figs. 7 and 8). It is noteworthy that comprehensive cDNA microarray analyses have identified common target genes between IkB-ζ and C/EBPβ in LPS-stimulated macrophages (13, 25). In addition, the binding sites for C/EBP proteins have been identified in the promoter regions of many IkB-ζ-regulated genes, and we have previously shown the importance of these sequences in the promoter activation (18). As expression of IkB-α(SR) suppressed the association of C/EBPβ with the Lcn2 promoter, but LIP expression did not perturb p65 recruitment, IkB-ζ recruitment of NF-κB seems to be a prerequisite for C/EBPβ recruitment.

Our ChIP analyses using two types of cells revealed that IkB-ζ is required for recruitment of the chromatin-remodeling factor Brg1 to the selective promoter (Figs. 7 and 8). As the experiment using CHX indicated that IkB-ζ is the only primary response gene necessary for the expression of its target secondary response genes (Fig. 3), the requirement of de novo protein synthesis for the Brg1-mediated nucleosome remodeling (7) could be explained by expression of IkB-ζ protein. During the preparation of this report, Kayama et al. (26) reported the involvement of MyD88-dependent signaling and IkB-ζ in selective expression of pro-inflammatory genes. As they showed that the promoter association of Brg1 and nucleosome remodeling at the target promoters are defective in MyD88-deficient cells but are normal in IkB-ζ-deficient cells, they hypothesized at least one other MyD88-dependent molecule that is required for Brg1-mediated nucleosome remodeling. Although the exact reason for the differences between this study and the published report regarding the role of IkB-ζ is unknown, there could be differences in regulation.

3 S. Yamazaki, unpublished observation.
Among IkB-ζ-dependent genes. Furthermore, we observed a cell type-specific requirement of IkB-ζ for induction of some genes. In this study, we focused on the target genes whose expression is highly dependent on IkB-ζ both in fibroblasts and macrophages. Although IL-6 production was severely impaired in IkB-ζ-deficient macrophages (13), the expression of IL-6 in response to TNF-α was not significantly augmented by zinc-induced IkB-ζ as that of Lcn2 and Csf3 (data not shown). As the TNF-α-induced IL-6 production in MEFs was shown to be unaffected by IkB-ζ deficiency (13), it is likely that IkB-ζ is not a limiting factor in this response.

Gene knockdown experiment using specific siRNAs indicated the close functional relationship between IkB-ζ and the NF-κB p50 subunit. We have previously shown a preferential physical interaction of IkB-ζ with p50 rather than other NF-κB subunits, and an overlap in the target genes of IkB-ζ and p50 (8, 13). The experiments using siRNA and Helenalin revealed that while p65 was necessary for induction of Lcn2, it was dispensable for Csf3 expression (Fig. 5). It is likely that p50 and IkB-ζ could form a core element for transcriptional activation of target genes (see below), and transcriptional activity of p65 might be required for full activation of some target genes. Because p50 does not contain a transactivation domain, transcriptional activity derived from other sources should be involved in the induction of Csf3, which is possibly mediated by IkB-ζ itself and C/EBP proteins (18, 27). Requirement of C/EBPβ for expression of Csf3 has been reported thus far (28).

It is still an enigma how IkB-ζ regulates the promoter accessibility of the key transcription regulators in a gene-selective manner. Because IkB-ζ has no obvious DNA binding motif, it is unlikely that IkB-ζ associates with the target promoter prior to the DNA-binding proteins. It is conceivable that IkB-ζ induced upon stimulation could form a complex with the p50 homodimer, which is known to be constitutively present in the specific promoters (13, 29). Then, this complex might act as a landmark for recruitment of Brg1, p65, and C/EBP. IkB-ζ could stabilize or assist the promoter binding of these transcription regulators by an unknown mechanism. Despite a careful comparison among the promoter sequences of IkB-ζ-regulated genes, no consensus structural features have been deduced thus far. Epigenetic factors, including covalent modifications of promoters and histones, could be a determinant of IkB-ζ dependence. Intriguingly, Kayama et al. (26) demonstrated the involvement of IkB-ζ in stimulus-dependent histone H3K4 trimethylation of the secondary response promoters. Along with the constitutively associated p50 subunit, low level of this

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