Stimulus-specific Induction of a Novel Nuclear Factor-κB Regulator, IκB-ζ, via Toll/Interleukin-1 Receptor Is Mediated by mRNA Stabilization*

We have recently identified an inducible nuclear factor-κB (NF-κB) regulator, IκB-ζ, which is induced by microbial ligands for Toll-like receptors such as lipopolysaccharide and the proinflammatory cytokine interleukin (IL)-1β but not by tumor necrosis factor (TNF)-α. In the present study, we examined mechanisms for stimulus-specific induction of IκB-ζ. The analysis of the IκB-ζ promoter revealed an essential role for an NF-κB binding sequence in transcriptional activation. The activation, however, did not account for the Toll-like receptor/IL-1 receptor-specific induction of IκB-ζ, because the promoter analysis and nuclear run-on analysis indicated that its transcription was similarly induced by TNF-α. To examine post-transcriptional regulation, we analyzed the decay of IκB-ζ mRNA, and we found that it was specifically stabilized by lipopolysaccharide or IL-1β but not by TNF-α. Furthermore, we found that costimulation with TNF-α and another proinflammatory cytokine, IL-17, elicited the IκB-ζ induction. Stimulation with IL-17 alone did not induce IκB-ζ but stabilized its mRNA. Therefore, IκB-ζ induction requires both NF-κB activation and stimulus-specific stabilization of its mRNA. Because IκB-ζ is essential for expression of a subset of NF-κB target genes, the stimulus-specific induction of IκB-ζ may be of great significance in regulation of inflammatory reactions.

Cells reprogram gene expression upon environmental changes to maintain homeostasis. For multicellular organisms, serious environmental changes include microbial infection, and a major rearrangement of gene expression occurs during inflammatory responses against infection. The expression of a variety of genes for inflammatory mediators is strongly activated upon stimulation with microbial and viral products that stimulate toll-like receptors (TLRs),1 and with inflammatory cytokines such as interleukin (IL)-1β or tumor necrosis factor (TNF)-α. Location, degree, and duration of expression of these genes are strictly regulated at the level of transcription, processing and stabilization of mRNA, and/or protein synthesis and degradation. Loss of the regulation could lead to severe disorders exemplified by systemic inflammatory response syndrome, rheumatoid arthritis, or scleroderma.

Nuclear factor-κB (NF-κB) is an evolutionarily conserved transcription factor that plays pivotal roles in inflammation as well as cell growth, survival, and differentiation (1–5). It consists of homo- and heterodimers of Rel/NF-κB family proteins with the Rel homology domain, which is implicated in both DNA binding and dimer formation. In resting cells, NF-κB is sequestered in the cytoplasm via association with cytosolic IκB proteins, IκB-α, -β, and -ε, through interaction between the ankyrin repeats of the IκB proteins and the Rel domains of NF-κB. Upon stimulation, the cytosolic IκB proteins are phosphorylated followed by ubiquitin/proteasome-mediated degradation, and NF-κB translocates into the nucleus where it engages in active transcription of the target genes. One of the NF-κB target genes is IκB-α, and the re-synthesized IκB-α binds to the nuclear NF-κB to export it to the cytoplasm and terminate the sequence of the reactions (1, 2, 4, 6). The activity of NF-κB is also modulated in the nucleus. Not only phosphorylation and acetylation of NF-κB itself (7, 8) but interactions with other nuclear proteins play critical roles in the regulation of the transcriptional activity of NF-κB. Modulators of the nuclear NF-κB include histone deacetylases (9), transcriptional coactivator and corepressor proteins (10), and other transcription factors (11–13).

Recently, we and others (14–16) have identified a new nuclear IκB protein, IκB-ζ (also named as MAIL or INAP), which interacts with NF-κB via a C-terminal ankyrin repeat domain in the nucleus (14). IκB-ζ is barely able to be detected in resting cells but is robustly induced in response to lipopolysaccharide (LPS). The induced IκB-ζ localizes in the nucleus and preferentially interacts with the NF-κB p50 subunit rather than the p65 subunit. The initial characterization of IκB-ζ has shown that it negatively regulates NF-κB activity, because transfection of IκB-ζ inhibits NF-κB reporter activity stimulated with LPS, IL-1β, or TNF-α, and inhibits activity induced by transfection of the NF-κB p65 subunit. On the other hand, subsequent functional analyses of the N-terminal region of IκB-ζ revealed that it had a latent activity of transcriptional activation.2 In addition, the most recent studies using IκB-ζ-deficient mice demonstrated that IκB-ζ is essential for the induction of a

tathione S-transferase, ORF, open reading frame; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ARE, AU-rich elements.

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‡ The abbreviations used are: TLR, toll-like receptor; IL, interleukin; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; IL-1, IL-1 receptor; TIR, Toll/IL-1R; MAP, mitogen-activated protein; TRAF, TNF receptor-associated factor; NGAL, neutrophil gelatinase-associated lipocalin; GRO, growth-related oncogene; GST, glutathione-S-transferase; ORF, open reading frame; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ARE, AU-rich elements.

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subset of the inflammatory genes including IL-6, the IL-12 p40 subunit, and granulocyte-macrophage colony-stimulating factor (17). The transcriptional activity of IκB-ζ depends on the p50 subunit, which exhibits target DNA binding activity without the transactivation activity (17).

Detailed analyses on the induction of IκB-ζ showed that not only LPS but also other microbial TLR ligands including peptidoglycan, the bacterial lipopeptides, and CpG DNA elicited IκB-ζ (17, 18). In addition to these microbial products, the proinflammatory cytokine IL-1β strongly induced IκB-ζ (14). TLRs and IL-1 receptor (IL-1R) share similar cytoplasmic domains known as Toll/IL-1R (TIR) domain and the common adaptor molecule MyD88 (19, 20). As expected, the induction of IκB-ζ by the TLR ligands is dependent on MyD88 (17). Prominent cellular responses triggered by activation of TLR/IL-1R include activation of NF-κB and mitogen-activated protein (MAP) kinases, and our previous studies have revealed that NF-κB is essential for IκB-ζ induction (18). Another proinflammatory cytokine TNF-α also activates both NF-κB and MAP kinases, which is similar to TLR ligands or IL-1β. However, IκB-ζ induction by TNF-α was marginal, indicating that NF-κB activation is not sufficient for the induction of IκB-ζ (14). Furthermore, overexpression of the NF-κB subunits resulted in robust induction of IκB-ζ but not IκB-ζ, leading to the conclusion that an additional signal derived from the TIR domain is required for IκB-ζ induction (18).

Although IL-1β and TNF-α do not share sequence homology with each other and they bind to distinct receptors, the intracellular signaling activated by the two cytokines converges to similar adaptor molecules, TNF receptor-associated factor (TRAF) 2 and TRAF6, each of which leads to the activation of NF-κB and MAP kinases (1, 21). Consequently, many of the target genes for IL-1β and TNF-α overlap, and the two proinflammatory cytokines give rise to similar biological effects exemplified by inflammation, coagulation, or pyrexia.

The biological effects, however, are not identical between the two cytokines. In addition to IκB-ζ, several other genes are preferentially induced by IL-1β but not by TNF-α. These genes are neutrophil gelatinase-associated lipocalin (NGAL) (22), the growth-related oncogene (GRO) homologs/celloc kinase (23, 24), the extracellular metalloprotease MMP-3 (25), and IL-6 (26–28). Conversely, expression of complement factor H was up-regulated by TNF-α but not by IL-1β (28). Promoter analysis of NGAL showed that it is preferentially activated by IL-1β rather than by TNF-α, implying that the differential induction is determined at the transcriptional level. Therefore, an IL-1β-specific transcriptional activator(s) or a TNF-α-specific repressor(s) exists (22). In fact, IκB-ζ has been shown to be one of the essential factors for expression of a subset of inflammatory genes such as IL-6 and NGAL (17). The NF-κB p50 subunit is required for the function of IκB-ζ, and only IκB-ζ is preferentially induced upon stimulation with IL-1β and TLR ligands but not with TNF-α. Therefore, IκB-ζ might play a role as a master gene that determines the expression of a subset of the inflammatory genes.

In the present study, we analyzed molecular mechanisms for the differential induction of IκB-ζ. We found that stability of IκB-ζ mRNA was specifically up-regulated by stimulation with LPS or IL-1β but not with TNF-α. This finding indicates that in addition to NF-κB activation, the other signal required for IκB-ζ induction, which comes from the TIR domain but not from the TNF-α receptor, leads to mRNA stabilization. Given the essential function of IκB-ζ in the expression of a series of inflammatory genes, the control of IκB-ζ expression by different stimuli may play a key role in determining the nature of inflammation.
TNF-α to induce IκB-ζ could be that a negative signal was specifically generated by TNF-α stimulation. In order to examine this possibility, we treated cells with LPS or IL-1β together with TNF-α. LPS- or IL-1β-stimulation of NIH3T3 cells resulted in the strong induction of IκB-ζ. Although TNF-α similarly induced other NF-κB target genes such as IκB-α and A20, TNF-α-stimulation of the cells induced little IκB-ζ, as described previously (Fig. 1A). Costimulation with TNF-α did not affect the LPS- or IL-1β-mediated induction of IκB-ζ (Fig. 1A), thus ruling out the possibility of a negative signal by TNF-α. The expression of IκB-ζ was dependent on de novo transcription because the induction of IκB-ζ by LPS or IL-1β was completely abolished by treating the cells with actinomycin D, an inhibitor of transcription. In the current conditions, the stimulation of NIH3T3 cells with LPS, IL-1β, and TNF-α activated NF-κB to a similar extent when measured with an NF-κB reporter, pELAM1-Luc (29, 31) (Fig. 1B). NF-κB activation by LPS or IL-1β, but not that by TNF-α, was specifically inhibited by expression of a dominant-negative mutant of MyD88, a TLR/IL-1R-specific adaptor molecule (19, 20). The stimulus-specific induction of IκB-ζ was also observed in A549 cells, which are a well-characterized human alveolar type II epithelial cell line (Fig. 1C). In addition, the stimulus-specific induction of IκB-ζ was observed in HEK293 or THP-1 cells (data not shown). This indicated that the preferential induction of IκB-ζ by activation of TLR/IL-1R is not cell type-specific and is conserved between mouse and human.

Induction of endogenous IκB-ζ protein was examined by immunoblotting with an antibody raised against a recombinant protein containing the C-terminal region of mouse IκB-ζ (anti-IκB-ζ(20)). An 85-kDa band that reacted with the antibody appeared 40 min after stimulation in LPS-treated RAW264.7 cells (Fig. 2A) and in NIH3T3 cells stimulated with LPS or IL-1β (Fig. 2B). A faint band of 70-kDa also appeared at ~60 min after the stimulation. In contrast to the cytosolic IκB proteins including IκB-α, -β, or -ε, IκB-ζ was barely detected in unstimulated cells, and rapid degradation after the stimulation was not observed. The IκB-ζ protein level peaked at 1–2 h after stimulation (Fig. 2A) and then gradually decreased (data not shown). Consistent with the mRNA induction, IκB-ζ protein was strongly induced by LPS or IL-1β, but we observed minimum induction by TNF-α (Fig. 2B). Immunoblotting with the anti-active p38 antibody showed that the level of p38 was barely detected in unstimulated cells, and rapidly increased after LPS treatment (Fig. 2B).

**FIG. 1.** IκB-ζ mRNA is induced in response to LPS or IL-1β but not to TNF-α. A, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for 1 h in the presence or absence of actinomycin D (5 μg/ml). Total RNA was extracted and subjected to Northern blot analyses with a probe for IκB-ζ, IκB-α, A20, or G3PDH. B, NIH3T3 cells were transfected with the pELAM1-Luc reporter plasmid and increasing amounts (0, 0.1, or 1.0 μg) of an expression vector for a dominant-negative form of MyD88 (MyD88C) together with pRL-TK. Two days after transfection, cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for 6 h, and the luciferase activities were measured. C, total RNA was extracted from A549 cells stimulated with IL-1β (50 pg/ml) or TNF-α (20 ng/ml) for 1 h and subjected to Northern blot analyses with the indicated probes.

**FIG. 2.** IκB-ζ protein is induced in response to LPS or IL-1β but not to TNF-α. A, RAW264.7 cells were stimulated with LPS (100 ng/ml) for the indicated periods. Total cell lysate was prepared and subjected to immunoblot analyses using antibodies against the C-terminal region of mouse IκB-ζ, active p38 (p-p38), and p38. B, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods, and total cell lysate was subjected to immunoblot analyses as described for A. C, total cell lysates from NIH3T3 cells transfected with an empty vector (vec), an expression vector for mouse IκB-ζ(L) (anti-IκB-ζ(LN)), and from RAW264.7 cells stimulated with LPS (100 ng/ml) for the indicated periods were subjected to immunoblot analyses using antibodies against the C-terminal region of IκB-ζ (anti-IκB-ζ(CL)) or the N-terminal region of IκB-ζ (anti-IκB-ζ(L)). The 70-kDa band corresponding to IκB-ζ(S) is indicated by an asterisk. A dot shows a nonspecific band.
IL-6 mRNA is strongly induced in response to LPS or IL-1β but not to TNF-α. A, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods. Total RNA was extracted and subjected to Northern blot analyses with a probe for IκB-ζ, IL-6, IκB-α, or G3PDH. B, total RNA from RAW264.7 cells stimulated with LPS (100 ng/ml) for the indicated periods was subjected to Northern blot analyses with the indicated probes.

The Preferential Induction of IκB-ζ Determines the Expression of the Target Gene—The analyses of IκB-ζ-deficient mice have revealed that IκB-ζ is essential for the expression of a subset of NF-κB target genes (17). Therefore, stimulus-specific induction of IκB-ζ should be reflected by the subsequent expression of these genes. NIH3T3 cells were stimulated under the same conditions as shown in Fig. 1. We then examined the induction of IL-6, a representative gene that requires IκB-ζ for expression. The expression of IL-6 was more strongly induced by LPS or IL-1β than by TNF-α, similar to that observed for IκB-ζ (Fig. 3A). The induction pattern was similar between IκB-ζ and IL-6, but the kinetics of the induction were different. IL-6 mRNA peaked at 2 h after stimulation with LPS or IL-1β in NIH3T3 cells, but it reached a peak at 4 h after stimulation in LPS-stimulated RAW264.7 cells (Fig. 3B). In either case, IκB-ζ was induced more rapidly than IL-6 and peaked at 1 h after the stimulation. These observations are consistent with the concept that IκB-ζ controls the expression of IL-6.

NF-κB Sites in the Promoter Are Essential for the Transcriptional Up-regulation of the IκB-ζ Gene—We obtained genomic fragments corresponding to mouse and human IκB-ζ promoter regions to analyze the transcriptional regulation of the IκB-ζ gene. Rapid amplification of cDNA ends analyses using poly(A)-tailed RNA from RAW264.7 cells or THP-1 cells stimulated with LPS identified several possible transcription initiation sites. The main initiation site is indicated by a box labeled as +1 in Fig. 4A. The nucleotide sequences around the transcription initiation site were well conserved between mouse and human and were preceded by a potential TATA box. The reporter analysis in RAW264.7 cells with serially deleted fragments of the mouse promoter region showed that reporters with promoter fragments containing a region spanning −359 bp to the initiation site were activated upon LPS stimulation. The minimal IκB-ζ promoter responded more strongly to LPS stimulation than an artificial promoter with four tandem copies of the NF-κB consensus sequence (4×κB). Thus, the cis-element(s) responsible for LPS responsiveness is present within −359 bp from the transcription initiation site (Fig. 4B).

We identified three canonical κB sites in the proximal promoter region of the mouse IκB-ζ promoter, and we designed them as κB1 (−256 to −247), κB2 (−218 to −209), and κB3 (−83 to −74). Although the κB1 and κB2 sites were completely conserved between the mouse and human IκB-ζ promoters, the κB3 sequence was poorly matched with the κB consensus sequence and was not conserved between the two species. We introduced a mutation into each of the sites to examine the contributions of these κB sites to the induction of IκB-ζ. The mutation at the κB2 site completely abolished the LPS-mediated promoter activation, whereas the mutation at the κB1 site resulted in moderate activation (Fig. 4B). In contrast, the mutation at the κB3 site did not affect the promoter activity.

In Additional to LPS and IL-1β, TNF-α Also Activates the Transcription of IκB-ζ—We investigated whether the LPS or IL-1β-specific induction of IκB-ζ was also observed by promoter analysis. NIH3T3 cells transfected with various reporters were stimulated as shown in Figs. 1–3. LPS, TNF-α, IL-1β similarly activated NF-κB, p38 MAP kinase, and the induction of the typical NF-κB target genes. Stimulation with LPS or IL-1β elicited modest activation of the IκB-ζ promoter (Fig. 5A). Unexpectedly, TNF-α induced similar or even stronger activation of the IκB-ζ promoter when compared with LPS and IL-1β. We examined the upstream regions up to −11 kb from the transcription initiation site but did not find any elements that conferred further responsiveness or LPS/IL-1β-specific up-regulation of the promoter activity (data not shown). The mutation at the κB2 site, but not that at the κB1 or κB3 sites, abolished the activation by all three stimuli, confirming the critical contribution of NF-κB in the induction of IκB-ζ.

We also tested the IκB-ζ promoter activity in human alveolar A549 cells. By titrating IL-1β and TNF-α, we determined the concentration of each stimulus that resulted in similar levels of activation of the NF-κB reporter. Stimulation of A549 cells with the determined concentrations of IL-1β and TNF-α exhibited an even higher activation of the IκB-ζ promoters by TNF-α than by IL-1β, regardless of length and species of the promoters (Fig. 5B). On the other hand, a promoter of human NGAL selectively responded to IL-β as reported by Cowland et al. (22).

We performed the nuclear run-on analyses to directly measure the transcriptional activity of the IκB-ζ gene in the stimulated cells. LPS, IL-β, and TNF-α elicited transcriptional activation of the IκB-ζ gene as well as the other NF-κB target genes.
genes, A20 and IxB-α. Except for the strongest activity observed 35 min after LPS stimulation, the transcriptional activity of the IxB-ζ gene was more strongly induced by TNF-α than by IL-1β or LPS, as observed in the promoter analyses (Fig. 6, A and B). The transient but strong transcriptional activation in the LPS-stimulated cells appeared to be commonly found with A20 and IxB-α, which are equally induced by the three stimuli in other cell types (Fig. 6, C and D). Thus, we concluded that the stimulus specificity of IxB-ζ induction is not determined at the transcriptional level.

LPS or IL-1β, but Not TNF-α, Specifically Stabilizes IxB-ζ mRNA—We investigated the stability of IxB-ζ mRNA in the presence of given stimuli in order to explore the post-transcriptional regulation of IxB-ζ expression. Because IxB-ζ mRNA can barely be detected in unstimulated cells, we constructed an expression plasmid to express the full-length IxB-ζ mRNA without stimulation. In the plasmid pAG-mIxB-ζ, a DNA fragment coding for IxB-ζ mRNA from -27 to +4005 was located downstream of a chicken β-actin promoter, which is a strong promoter unresponsive to the stimuli. A stable cell line established with the plasmid constitutively expressed the full-length mRNA for IxB-ζ (Fig. 7A). The kinetics of the decay of the mRNA derived from the IxB-ζ transgene and the endogenous mRNA for IxB-α were analyzed after treating the cells with actinomycin D to terminate de novo transcription (Fig. 7, B and C). In the absence of stimuli, IxB-ζ mRNA was degraded with a half-life of about 30 min, in a similar manner to IxB-α. This is in contrast to the stable mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Most interestingly, the degradation of the IxB-ζ mRNA was specifically delayed by stimulation with LPS or IL-1β but not with TNF-α. LPS or IL-1β extended the half-life of the IxB-ζ mRNA more than three times than that without stimuli. In contrast to IxB-ζ mRNA, the half-life of IxB-α mRNA was not affected by the stimuli. These results indicated that IxB-ζ mRNA is specifically stabilized upon LPS or IL-1β stimulation.

A cis-Element in IxB-ζ mRNA Is Responsible for the Stimulus-specific Stabilization—To identify a cis-element(s) for the stimulus-specific stabilization of IxB-ζ mRNA, we replaced the ORF of IxB-ζ in the expression vector with that of the luciferase gene (Fig. 8A, ρ-Luc-ζ). NIH3T3 cells were transfected with the plasmid, and the decay of the chimeric mRNA was chased following treatment with actinomycin D. The stimulus-specific stabilization of mRNA was not observed with the chimeric mRNA (Fig. 8B, upper panel), suggesting that replacement of the ORF resulted in deletion of the cis-element(s) responsible for the stabilization. We attempted to identify the cis-element(s) within the ORF of IxB-ζ. The ORF was divided by an internal NcoI restriction site into a 765-bp fragment coding for the N-terminal (IxB-ζ (N)) and a 1,421-bp fragment for the...
C-terminal (IκB-ζ (C)) regions. Each of the fragments was inserted upstream or downstream of the luciferase ORF in the ζ-Luc-ζ constructs (Fig. 8A). The resulting chimeric mRNA ζ-ζ-Luc-ζ harboring the 765-bp N-terminal region was specifically stabilized by LPS and IL-1β, whereas that with the C-terminal region, ζ-Luc-ζ-ζ, did not respond to these stimuli (Fig. 7B, the middle and the bottom panels). We tried to further determine the cis-element(s), but all the constructs with the N-terminal region shorter than 765 bp failed to show the stimulus-specific stabilization by LPS/IL-1β (data not shown). Thus, the cis-element responsible for the stimulus-specific stabilization is in the 765-bp N-terminal region of the ORF of IκB-ζ.

**IL-17 Generates an mRNA Stabilization Signal to Induce IκB-ζ in Combination with TNF-α**—The proinflammatory cytokine IL-17 has been shown to augment the induction of a subset of inflammatory genes by TNF-α. Most intriguingly, several groups (33–35) have recently reported that costimulation with TNF-α and IL-17 up-regulates expression of IL-6 and the GROα homolog. In order to examine the effect of IL-17 on IκB-ζ induction, NIH3T3 cells were stimulated with IL-17 alone or in combination with TNF-α or IL-1β. Although marginal expression of IκB-ζ mRNA was observed in the cells stimulated with IL-17 alone, costimulation with TNF-α and IL-17 elicited robust IκB-ζ induction comparable with that by LPS or IL-1β (Fig. 9A). On the other hand, the induction of IκB-α was not affected by stimulation of IL-17 alone or in combination with TNF-α. IL-17 did not affect the IL-1β-mediated induction of IκB-ζ. The kinetics of IκB-ζ induction by TNF-α and IL-17 was similar to that by LPS or IL-1β (Fig. 9B).

The activation of NF-κB and p38 MAP kinase was examined in order to identify the signal(s) generated by IL-17 stimulation. The degradation of IκB-α in the cytoplasm is preceded by activation of NF-κB. Immunoblotting analysis with anti-IκB-α antibody revealed the transient degradation of IκB-α followed by re-synthesis on stimulation with LPS, TNF-α, and IL-1β (Fig. 10A, top). However, the degradation was not detected with IL-17 stimulation, indicating that the NF-κB-activation signal is not significantly activated by IL-17. The NF-κB reporter analyses showed that there was no NF-κB activation with increasing amounts of IL-17, further supporting this conclusion (Fig. 10B). Immunoblotting with anti-p38 antibody showed that p38 MAP kinase was activated by LPS, TNF-α, or IL-1β but not by IL-17 (Fig. 10A, middle).

We finally examined the effect of IL-17 on the stabilization of IκB-ζ mRNA using the stable cell line. As expected, the decay of IκB-ζ mRNA was delayed in response to IL-17 alone as well as LPS or IL-1β (Fig. 10C). Therefore, IL-17 alone provided a stabilizing signal for IκB-ζ mRNA, which was lacking in the cells stimulated with TNF-α. Thus, costimulation with TNF-α and IL-17 induces IκB-ζ by compensating the two distinct signals required for the induction, namely activation of NF-κB and the specific stabilization of IκB-ζ mRNA.

**DISCUSSION**

Our previous studies have indicated that activation of NF-κB is essential but not sufficient for the induction of IκB-ζ (14, 18). Another different signal originated from TLR/IL-1R and was missing in the cells stimulated with TNF-α. This signal is required for induction of IκB-ζ, but its identity has remained unknown. In the present study, we attempted to find the signaling required for the differential induction of IκB-ζ by IL-1β or LPS. The promoter analyses revealed that the NF-κB-binding sites were critical for the induction of IκB-ζ, supporting our conclusion above from the previous studies (18). However, neither the promoter analyses nor the nuclear run-on assay provided any evidence for preferential transcription of the IκB-ζ gene. Instead, we found that IκB-ζ mRNA was preferentially stabilized by IL-1β and LPS but not by TNF-α.

We also attempted to identify certain stimuli that complement the missing signal in the TNF-α signaling. After extensive searching, we identified IL-17, which in combination with TNF-α activated the induction of IκB-ζ. The stimulation with IL-17 alone did not induce IκB-ζ but stabilized IκB-ζ mRNA without significant activation of NF-κB and p38 MAP kinase. We also found that IL-17 augmented the TNF-α-mediated NF-κB reporter activity but not the IL-1β-mediated activity (data not shown). However, simple augmentation of the NF-κB activity induced by TNF-α is unlikely to account for the compensating action of IL-17, because overexpression of the NF-κB p65 subunit resulted in remarkable NF-κB activity but did not induce IκB-ζ (18). Therefore, it is likely that costimulation of TNF-α and IL-17 induced IκB-ζ by activating the two separable signals required for the induction, which are NF-κB activation and mRNA stabilization.

The half-life of IκB-ζ in unstimulated cells was almost the same as that of IκB-α. This finding suggests that IκB-α mRNA is also unstable with a short half-life. Therefore, expression of IκB-α requires strong transcriptional activation or the stabilization signal for IκB-α mRNA. The stabilization of mRNA is
specific to IkB-ζ mRNA but not to IkB-α mRNA. Transcriptional up-regulation of IkB-ζ is not sufficient for induction, whereas IkB-α was strongly induced without the stabilization of mRNA. As expected from these findings, the transcription of IkB-α was much more strongly induced than that of IkB-ζ (Fig. 6A). Thus, induction of IkB-α appears to be simply regulated by strong activation of transcription but that of IkB-ζ requires at least two different signals, NF-κB-mediated transcriptional activation and mRNA stabilization, which could provide more delicate mechanisms for regulation of IkB-ζ induction.

A growing body of evidence suggests that proinflammatory stimuli induce stabilization of mRNAs encoded by a series of inflammatory genes to up-regulate their expression, as well as transcriptional activation (21, 36). For instance, mRNAs for principal inflammatory mediators such as cyclooxygenase 2, TNF-α, IL-6, and the chemokine GROα homolog have been shown to be stabilized in response to LPS or IL-1β stimulation (21, 23, 24). The involvement of the p38 MAP kinase cascade in the stabilization of these mRNAs has been suggested previously (21, 37–40). However, in the present study the stabilization of IkB-ζ mRNA did not appear to be dependent on p38 MAP kinase because TNF-α induced the activation of p38 MAP kinase to a similar extent as LPS or IL-1β (Figs. 2 and 10A). In addition, an inhibitor for the kinase, SB203580, did not affect

![Figure 6](image6.png)

**Fig. 6.** The transcription of the IkB-ζ gene is activated to a similar extent in response to LPS, IL-1β, and TNF-α. The transcription-competent nuclei were prepared from NIH3T3 cells (A) or RAW264.7 cells (C) stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods and were subjected to nuclear run-on analysis using a probe for vector, G3PDH, A20, IkB-ζ, or IkB-α, spotted onto nylon membranes. The activity was normalized with that of G3PDH. B and D, the radioactivity of each spot in A and C was quantitated, and the transcriptional activity of each gene was plotted.

![Figure 7](image7.png)

**Fig. 7.** IkB-ζ mRNA was stabilized in response to LPS or IL-1β but not to TNF-α. A, a schematic illustration of the construct for expression of the full-length IkB-ζ mRNA. A fragment for the full-length IkB-ζ mRNA is located downstream of the chicken β-actin promoter. A hatched box and closed ovals in the 3’-untranslated region (UTR) indicate the ORF of IkB-ζ and the AREs, respectively. The polyadenylation signal followed by the G/U-rich and U-rich sequences is shown in a shaded box. B, NIH3T3 cells stably expressing the full-length IkB-ζ mRNA were treated with actinomycin D (5 μg/ml) together with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods. Total RNA was extracted and subjected to Northern blot analyses with a probe for the IkB-ζ transgene (Tg), IkB-α, or G3PDH. C, the radioactivities in B were quantitated and shown after they had been normalized with those of G3PDH.
The induction of IκB-ζ (data not shown). Thus, molecular mechanisms underlying the stabilization of IκB-ζ mRNA and other gene products could be different.

We first expected that the cis-element(s) responsible for the stabilization of IκB-ζ mRNA would be present in either the 5′- or 3′-untranslated regions. The 3′-untranslated region of IκB-ζ mRNA harbors four AU-rich elements (AREs), which are known to destabilize mRNA. We examined the role for the AREs in the stability of IκB-ζ mRNA, but mutations in the elements did not severely affect the decay kinetics or the preferential stabilization (data not shown). We further attempted to analyze the effect of the trans-acting factors that stabilize mRNA by binding to the AREs, such as HuR and Apobec-1 (39, 41–44). However, overexpression of HuR or Apobec-1 failed to induce IκB-ζ mRNA, even after TNF-α stimulation (data not shown). The analyses of the chimeric mRNAs identified the cis-element in the region corresponding to the ORF. There have been several reports that showed the determinants for mRNA stability in the coding region (45–47).

FIG. 8. The cis-element(s) responsible for the stimulus-specific induction of IκB-ζ is in the ORF of IκB-ζ mRNA. A, schematic illustrations of the constructs for the chimeric mRNA composed of IκB-ζ and luciferase. B, NIH3T3 cells were transiently transfected with the indicated expression plasmids and treated with actinomycin D (5 μg/ml) together with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods. Total RNA was extracted and subjected to Northern blot analysis with a probe for luciferase, IκB-α, or G3PDH. The radioactivities were quantified and shown after they had been normalized with those of G3PDH.

FIG. 9. Costimulation with IL-17 and TNF-α induced IκB-ζ. A, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-1β (10 ng/ml), or increasing concentrations (2, 20, or 200 ng/ml) of IL-17 alone or in combination with TNF-α and IL-1β for 1 h. B, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-17 (20 ng/ml), IL-1β (10 ng/ml) alone, or TNF-α plus IL-17 for the indicated periods. Total RNA was extracted and subjected to Northern blot analyses with a probe for IκB-ζ, IκB-α, or G3PDH.

IL-17 is a proinflammatory cytokine produced exclusively from activated T cells (48–50). In contrast to its restricted expression, the receptor for IL-17 is expressed in a wide variety of cell types. Recent progress on genome-wide sequencing has identified several IL-17-related genes (IL-17 B to F) with redundant biological activities. IL-17 receptor is a type I transmembrane cell surface protein without significant homology to other known proteins (48, 50), and its intracellular signaling is poorly understood. Thus, mechanisms for IL-17-mediated mRNA stabilization remain to be investigated. Because mRNA stabilization was measured in the presence of actinomycin D, de novo synthesis of the mediators is not required for IL-17-mediated stabilization. Al-
FIG. 10. IL-17 stimulation stabilizes IκB-ζ mRNA. A, NIH3T3 cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), or IL-1β (10 ng/ml) for the indicated periods. The total cell lysate was prepared from the cells and subjected to immunoblot analyses using antibodies against anti-IκB-ζ, active p38 (p-p38), and β-tubulin. B, NIH3T3 cells were transfected with pELAM1-luc and pRL-TK. Two days after transfection, cells were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-1β (10 ng/ml), or the indicated concentrations of IL-17 for 6 h, and the luciferase activities were measured. C, NIH3T3 cells stably expressing the full-length IκB-ζ mRNA were treated with actinomycin D (5 μg/ml) together with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-17 (20 ng/ml), or IL-1β for the indicated periods. Total RNA was extracted and subjected to Northern blot analyses with a probe for the IκB-ζ transgene (Tg), IκB-α, or G3PDH.

Although IL-17 has been reported to activate NF-κB and MAP kinases (35, 51), these activities were not detected under our current experimental conditions. It has been suggested that IL-17 signaling requires TRAF6 but not TRAF2 (52, 53). Therefore, IL-17 signaling may be qualitatively similar to that of TLR/IL-1R. Our observation that IL-17 augmented the TNF-α-mediated NF-κB activation, but not the IL-1β-mediated activation, appears consistent with this possibility.

Although IL-17 has been reported to elicit the production of inflammatory mediators by itself, recent studies (33–35) have focused on the synergistic effects of IL-17 and TNF-α on the expression of chemokines and cytokines, including IL-6 and the chemokine GROα homolog. The stabilization of IL-6 mRNA (35) and the induction of the CCAAT/enhancer-binding protein (C/EBP)δ that engages in the transcription of IL-6 (34) have been shown to be synergistic effects. However, the induction of IκB-ζ by costimulation with TNF-α and IL-17 is likely to be involved in the induction of IL-6, because IκB-ζ is essential for the TLR/IL-1R-mediated induction of IL-6 (17).

The physiological significance of IκB-ζ induction by collaboration between TNF-α and IL-17 is currently unknown. However, a critical role for IκB-ζ in expression of a diverse array of genes raises various possibilities. Recent reports including the present study have suggested that T cell-derived IL-17 and proinflammatory cytokines produced by monocytes/macrophages such as TNF-α can cooperatively mediate inflammation. Collaboration between TNF-α and IL-17 has been shown to regulate the production of inflammatory cytokines or tissue remodeling factors from synoviocytes (49). IκB-ζ might play a crucial role in the induction of inflammatory genes in synovial tissues, which are closely associated with pathophysiology of rheumatoid arthritis or osteoarthritis.

In addition to the efforts to elucidate molecular mechanisms of IκB-ζ-mediated transcription, investigation of the molecular machinery that leads to IκB-ζ induction could be crucial to understanding the precise regulation of inflammation and pathological conditions derived from disorders of this regulation.

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