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Editor

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IkBz

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Synonyms

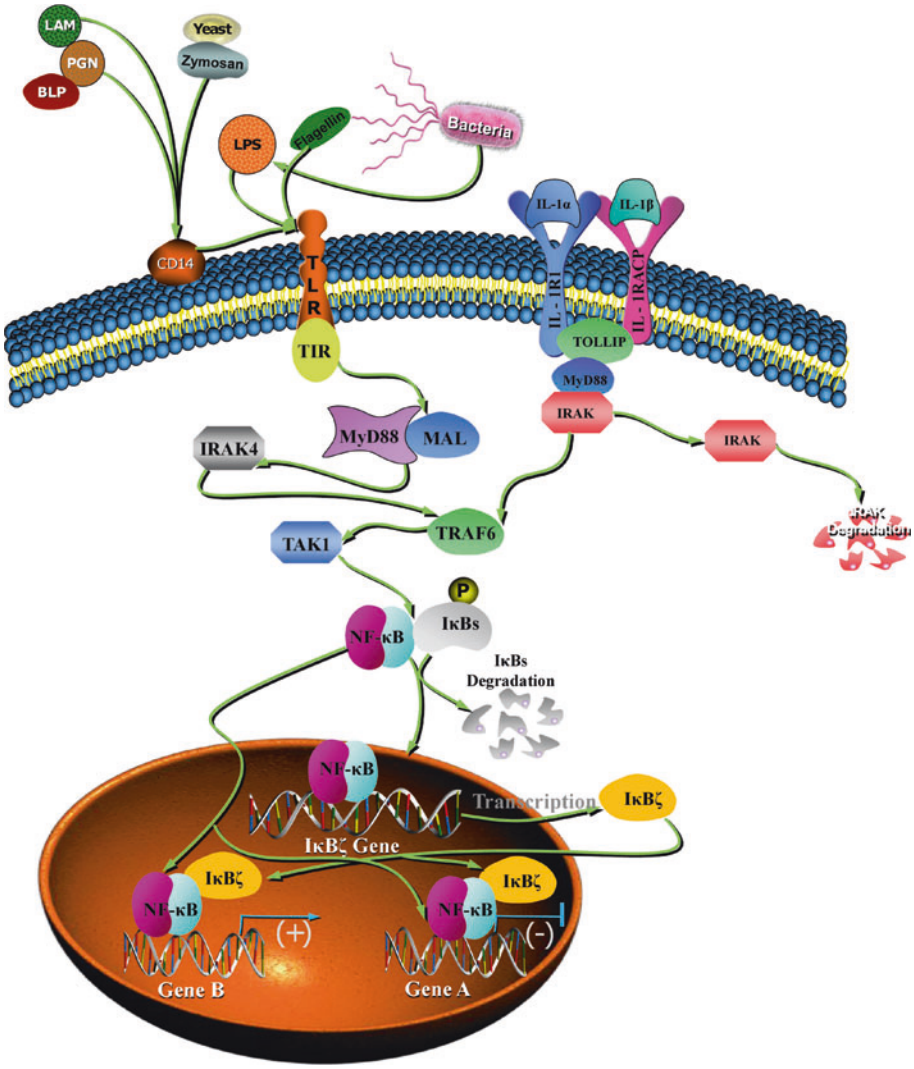
IkappaBzeta; IkB-zeta; IL-1 inducible nuclear ankyrin-repeat protein (INAP); Inap; Mail; Molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL); Molecule possessing ankyrin-repeats induced by lipopolysaccharide; NF-kappa-B inhibitor zeta; NFKBIZ; Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta

Historical Background

IκBζ is also known as molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL) or interleukin (IL)-1 inducible nuclear ankyrin-repeat protein (INAP) and it was first discovered independently in two laboratories during 2000 as a protein containing ankyrin repeats. The gene encodes a protein with an amino-terminal region of 450 amino acids that contains a nuclear localization signal (NLS) and a transactivation domain (TAD) followed by the seven recognizable ankyrin repeats at its carboxyl terminal. Treatment of cells with lipopolysaccharides (LPS) or the cytokine interleukin (IL)-1 (Kitamura et al. 2000; Haruta et al. 2001; Yamazaki et al. 2001) induced the expression of IκBζ, whereas tumor necrosis factor (TNF)-α (Totzke et al. 2006) treated cells did not show any IκBζ expression. The induced IκBζ is localized in the nucleus, where it interacts with the nuclear factor (NF)-κB subunit and other nuclear proteins via their ankyrin repeat domain (ARD), leading to a positive or negative regulation of its transcriptional activity depending on genes (Muta 2006). Thus, the innate immune system utilizes NF-κB as a major transcription factor and modulates its activity in a gene-specific manner via the regulatory factor IκBζ, which is specifically induced upon stimulation of the innate immune system. This multistep regulation of the transcription would be fundamental in the selective expression of genes upon cell activation. In this chapter, we summarize recent findings in nuclear IκBζ with an emphasis on its immunological aspects.

Induction of IκBζ and Its Functions

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 cytokine/chemokine, antimicrobial peptides, and cell adhesion molecules; most of the genes are known to be induced through activation of transcription factor NF-κB (Akira and Takeda 2004; Hoffmann and Baltimore 2006; Hayden and Ghosh 2008). In resting cells, typical cytoplasmic IκB proteins (IκB-α, -β, and -ε) mask the NLS of NF-κB, thereby preventing its translocation into the nucleus. The activation of cells with appropriate stimuli, particularly toll-like receptor (TLR) ligands or various host immune mediators such as proinflammatory cytokines and IL-1 superfamily proteins, induces activation of IκB kinase complex, which leads to the degradation of the cytoplasmic IκBs by the ubiquitin-proteosomal pathway. The NF-κB liberated from the IκBs is then translocated to the nucleus, where it binds to the promoter/enhancer region of the target genes, resulting in the regulation of transcription via recruitment of several coactivators and corepressors. This transcriptional activation leads to the expression of primary/early response gene A, depicted in Fig. 1, which includes three



IkBz, Fig. 1 Roles of IκBζ in inflammatory response. Activation of the TIR-containing receptors by TLR ligands elicits phosphorylation and ubiquitination-induced degradation of the cytosolic IκB proteins, which allows nuclear translocation of NF-κB. In the nucleus, NF-κB activates transcription of a subset of genes A, which includes IκBζ. The expression of IκBζ also requires a specific mRNA stabilization signal that comes from the TIR-containing receptor as well as activation of NF-κB. The expressed IκBζ associates with NF-κB, and the complex engages transcription of another subset of genes B. Simultaneously, IκBζ inhibits transcription of the subset of genes A

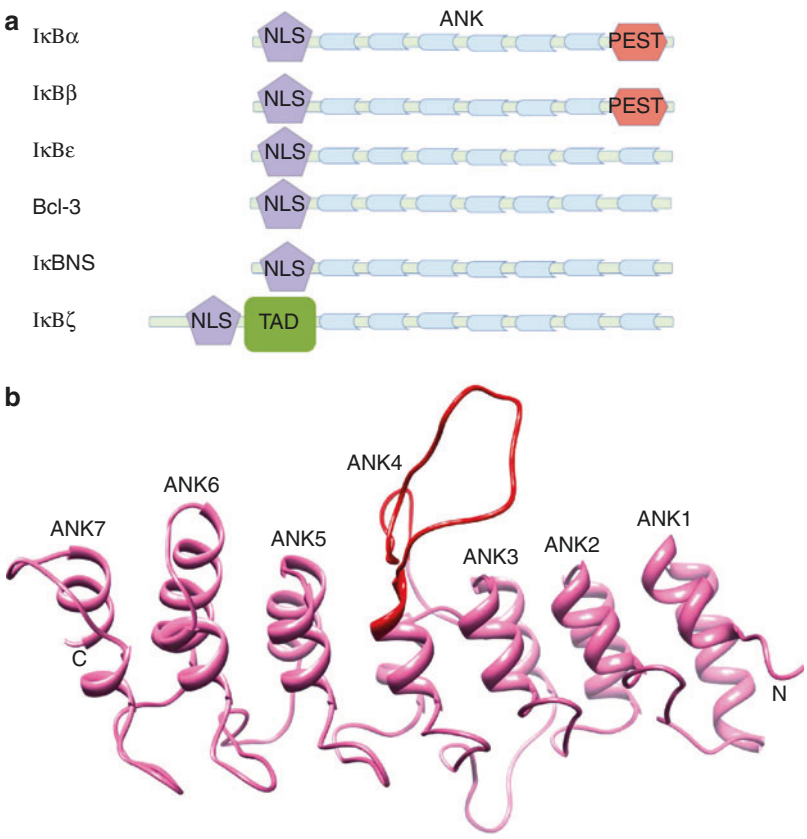
atypical members, IκBζ, Bcl-3, and IκBNS. The induced IκBζ associates with NF-κB and then activates another subset of inflammatory gene B (Fig. 1). Simultaneously, IκBζ inhibits the transcriptional regulation of gene A.

The predominantly expressed cytoplasmic IκB proteins, IκB-α, -β, and -ε, act exclusively as NF-κB inhibitors, whereas the nuclear IκB proteins, Bcl-3, IκBNS, and IκBζ, can both act as either a positive or negative regulator of NF-κB target genes. Bcl-3 can act as a positive regulator of NF-κB either by removing transcriptionally inactive p50 and p52 dimers from the IκB sites, thus allowing transcriptionally active heterodimers to take their places, or by forming a ternary complex with DNA-binding p50 and p52

homodimers, thereby facilitating gene expression (Fujita et al. 1993; Dechend et al. 1999; Yamazaki et al. 2001; Yamamoto et al. 2004; Totzke et al. 2006). IκBNS enters the nucleus where it preferentially interacts with p50 and functions as the repressor of NF-κB. Unlike other classical IκB proteins, IκBζ is strongly expressed in response to treatment with different proinflammatory stimuli. This list includes LPS, IL-1, peptidoglycan, bacterial and mycoplasmal lipopeptides, flagellin, R-848 (an imidazoquinoline derivative) as well as CpG oligonucleotides, and ligands for TLR2, -5, -7, and -9. The physiological role of IκBζ has been shown either as inhibiting the transcriptional activity of NF-κB by associating with the p50/p65 heterodimer or activating the expression of genes such as IL-6, CCL2, IL10, Lcn2, and IL-12p40 as binding with the p50 homodimer.

IκBζ is encoded by a primary responsive gene, *Nfkbiz*, and its induction depends on NF-κB activation, suggesting that IκBζ-regulated genes are induced via two-step machinery (Motoyama et al. 2005; Yamazaki and Takeshige 2008). Very little IκBζ is detected in unstimulated cells, and it is induced by various microbial substances that stimulate TLRs and IL-1β receptors but not by TNF-α. Actually, TNF-α induces transcription of *Nfkbiz* gene, but IκBζ mRNA did not stabilize during posttranscriptional process, indicating that the stimulus-specific expression of IκBζ is determined posttranscriptionally. All TLRs possess ectodomains that recognize ligands from microbial substances and endodomains that facilitate the downstream signaling, which has been shown to be associated with the ectodomain through a transmembrane segment. With the exception of TLR3, which mediates downstream signaling through TRIF/TRAM (which is Myd88 independent), the other TLRs share common signaling pathways initiated by an adaptor protein. The signal either through the Myd88 (Myd88-dependent pathway) or TRAF6 or both leads to the induction of IκBζ. In macrophage cells, LPS stimulation leads to three variants (L, S, and D) that are generated by alternative splicing of IκBζ. The longer form, IκBζ (L, 1–728), is predominantly expressed upon LPS stimulation, while the shorter form IκBζ (S) has been observed at mRNA and protein levels in minor species. Although IκBζ (D) mRNA has been detected in macrophages, its corresponding protein level has not been found (Yamazaki et al. 2005). Overall, IκBζ (L and S) are functionally active when expressed in the cells.

IκBζ (1–728 amino acids) can be divided into N-terminal (1–450) and C-terminal (451–728) portions. The N-terminal region composed of NLS and TAD, whereas the C-terminal region contains the ANK repeat domain, which plays an important role in interaction with NF-κB subunits, thereby regulating its functions (Fig. 2a). Truncated mutation studies of IκBζ (especially its N-terminal region) have shown -K $_{163}$-R $_{164}$-X $_{12}$-K $_{177}$-R $_{178}$- to be indispensable for NLS, and the mutation of this portion has revealed that IκBζ localized in the cytosol and effectively inhibited NF-κB, whose function is similar to the cytosolic IκB proteins. IκBζ was initially characterized as a negative regulator of NF-κB, but subsequent studies demonstrated that it could also act as a positive regulator of NF-κB. Analysis using GAL4-fusion protein of IκBζ revealed that its N-terminal region (329–403) exhibits transcriptional activity after association with NF-κB (p50/p50) subunit (Yamazaki et al. 2005). To date, numerous functions of IκBζ have been reported, when bound to other nuclear proteins: (1) Overexpression of IκBζ augmented IL-6 production in response to LPS by interacting with p50 homodimer, whereas TNF-α production is inhibited through interaction with p50/p65 heterodimer, indicating specific target gene activity (Motoyama et al. 2005). (2) IκBζ physically and functionally interacts with STAT3, which is a member of the NF-κB signaling pathway, thereby inhibiting the transcriptional activation of STAT3 (Wu et al. 2009). (3) Human IκBζ expressed in response to TNF-α binds to the DNA-binding region of p50/p65 heterodimer, consequently leading to the inhibition of the TNF-α response (Totzke et al. 2006). (4) IκBζ mediates preinitiation complex assembly and histone H3K4 methylation, leading to the activation of secondary response genes, thereby suggesting a role of IκBζ in the nucleosome remodeling (Kayama et al. 2008). (5) IκBζ is expressed in IL-17-producing helper T (T_H17) cells that play



IkBz, Fig. 2 IκB family members. (**a**) All IκB proteins harbor ankyrin repeats at their carboxy terminal region. The N-terminal regulatory region of IκB-α, -β, and -ε contains specific sequences that are phosphorylated and ubiquitinated. The N-terminal region of IκBζ contains the nuclear localization and transactivation domains that are necessary for the transcriptional process. (**b**) The predicted 3D model of the IκBζ ARD domain are shown in *bright pink*. The 28 amino acid residues occurring within the ANK4 are shown in *red*

an important role in resistance to experimental autoimmune encephalomyelitis (EAE) (Okamoto et al. 2010). (6) IκBζ controls the proliferation and differentiation of epidermal keratinocytes through NF-κB-independent mechanisms (Ishiguro-Oonuma et al. 2015).

Physiological Roles of IκBζ

Studies of IκBζ-deficient mice have demonstrated that IκBζ plays a role as a positive and negative regulator of NF-κB-mediated transcription (Yamamoto et al. 2004). Microarray studies have shown that IκBζ is an indispensable component of the LPS-induced transcription of genes represented by IL-6 and of the genes listed in Table 1. It should also be noted that IL-6 plays an important role in many inflammatory diseases including sepsis, heart attacks, and stroke as well as in many human cancers including hepatocarcinoma, multiple carcinoma, and ovarian cancer. However, the transcription complex on IL-6 appears to differ depending on the stimuli. The role of IκBζ as a negative regulator was not evident in the isolated cells, probably because of redundant negative regulators of NF-κB. The inhibitory roles of IκBζ in NF-κB-mediated transcription are critical in fine-tuning to balance inflammatory reactions to maintain homeostasis in vivo. IκBζ knockout animals have atopic-like dermatitis and eye inflammation that supports a role of IκBζ in innate host defense. However, the inflammation caused by IκBζ deficiency is not so clear. Accordingly, it will be important to test whether the skin and eye inflammation in IκBζ knockout mice is due to the lack of host defense molecules such as lipocalin or lack of essential cytokines such as IL-6. However, the other function of IκBζ, suppression of NF-κB activity, cannot be excluded. Atopic dermatitis and ocular inflammation may also occur due to the

IkBz, Table 1 Genes that require IκBζ for LPS-mediated induction

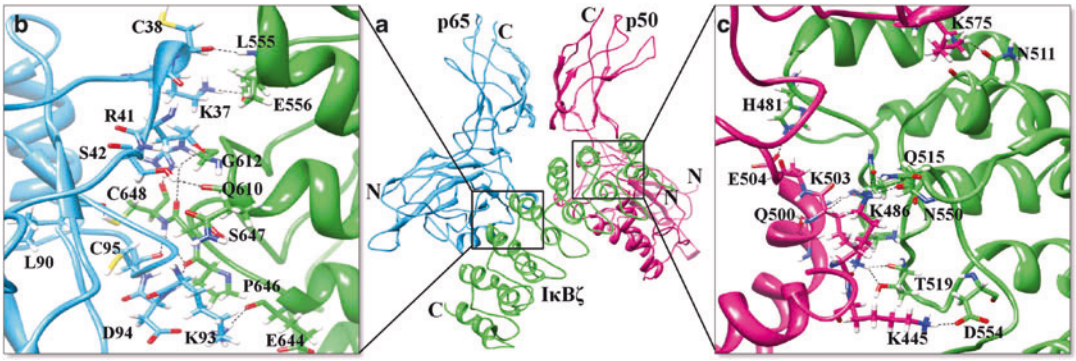
Category	Subset of genes
Cytokines	IL-6, IL-12 p40 subunit, IL-18, IL10, granulocyte macrophage stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), growth-differentiation factor (GDF) 15, Epstein-Barr-virus-induced gene (EBI) 3
Chemokines	CXC chemokine ligand (CXCL)5, CXCL13, chemokine ligand (CCL)7, CCL17, CCL2
Enzymes	Histidine decarboxylase, caspase11, inositol polyphosphate-5-phosphatase B, deltex 2B, glutathione reductase, guanylate nucleotide-binding protein (GDP) 1
Receptors	Formyl peptide receptor 1, macrophage receptor with collagenous structure (MARCO)
Biological active peptides	Endothelin 1, ghrelin
Transcription factors	Basic leucine zipper transcription factor (BATF), CCAAT/enhancer-binding protein (C/EBP)-δ
Antimicrobial substances	Lipocalin 2/neutrophil gelatinase-associated lipocalin (NGAL), Pentraxin 3
Others	Tax-1 binding protein, extracellular proteinase inhibitor, solute carrier family 11 member 2 (S1c11a2), Src-like adaptor protein (SLAP), immunoglobulin heavy chain, immunoglobulin light chain, membrane spanning 4-domains (MS4A1), thrombospondin 1, immediate early response 3 (IER3/IEX1), disabled-2

overexpression of cytokines that IκBζ might inhibit via the C-terminal ankyrin repeat.

The factors that distinguish genes that are activated or inhibited by IκBζ appear to be present in the promoter of each gene. Biochemical studies have indicated that in addition to NF-κB binding sites, IκBζ is also required for transcriptional activation. Since the transcriptional activation activity of IκBζ on the GAL4-reporter system is much weaker than that of the NF-κB p65 subunit, other transcription factors are necessary for efficient IκBζ-mediated transcription (Yamazaki et al. 2008). IκBζ acts as a negative regulator of the promoter harboring canonical NF-κB-binding sequences alone. Moreover, all the nuclear IκB proteins are homologous to each other; therefore, these nuclear proteins may act as competitors for IκBζ or vice versa. In fact, IκBNS has been reported to inhibit LPS-mediated IL-6, IL-12 p40, and IL-18 production (Kuwata et al. 2006).

Structure-Based Activation and Inhibition Mechanism of Nuclear IκBζ

The primary sequence of human and mouse IκBζ share about 70% homology with the N-terminal region (1–450) and 97% sequence identity with the C-terminal ARD. Although there is sequence variation at the N-terminal region, the NLS and TAD are conserved. There has been speculation about the sequence variations in the N-terminal region between human and mouse. IκBζ may play a significant role to bind with specific NF-κB subunits. Mouse IκBζ binds only to p50/p50 homodimer, whereas human IκBζ binds to both the p65 and p50 subunits. Sequence analysis of the N-terminal region did not identify any sequences known to correlate with specific structure or function. Moreover, secondary structure prediction showed that the N-terminal has no ANK repeat followed by the C-terminal ARD. This type of architecture has also been reported in proteins such as the yeast ribosomal binding protein yar-1 (Lycan et al. 1996). No crystal structure is yet available for IκBζ ARD, but recent modeling studies have shown the IκBζ three-dimensional structure, which was built based upon the Bcl-3 crystal structure (Michel et al. 2001) (Fig. 2b). Each ANK repeat of the IκBζ models depicted two antiparallel α-helices, followed by a loop of variable length at a right angle. Each repeat began and ended with short β-hairpin turns that protruded away from the α-helix. This nonglobular fold was stabilized through intra- and interrepeat hydrophobic interactions. The represented structural motifs stack

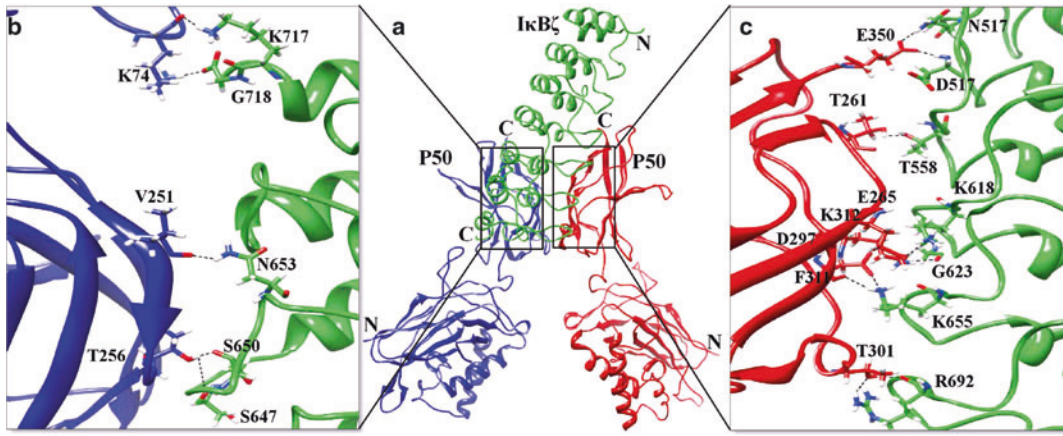


IkBz, Fig. 3 Docking studies predicted that the IκBζ ARD binds at the side of the p50/p65 heterodimer interface. (**a**) The p50/p65 heterodimers, represented as a ribbon diagram, are shown in *magenta* and *cyan*, respectively. Docked IκBζ is *green* in the ribbon diagram. (**b**) p65-IκBζ binding interface. Side chains of the amino acid contributing to the hydrogen bonding formation (marked as *black*) are represented by a stick model with the residue name and numbers shown next to them. (**c**) The p50-IκBζ binding interface is shown in a similar fashion as in (**b**)

upon one another in a linear fashion to form a curved architecture. These motifs are known to facilitate protein–protein interaction but have no known enzymatic activity (Mosavi et al. 2004). The presence of ankyrin repeats and their role in inflammatory signaling immediately suggested structural and functional homology with the ARD-containing classical IκB proteins. Despite the similarities, IκBζ differs structurally from the classical IκB inhibitors in its unique amino-terminal region, the number of ankyrin repeats, the presence of a unique 28 amino acids insertion within the helices of the α1 and α2 of ANK4 (similar to one that has been observed in IκBNS), and the complete absence of a PEST-like region within the IκBζ carboxy-terminus (Fig. 2b).

Recent studies have indicated that human TNF-α-induced IκBζ is associated with both the p50/p65 subunits of NF-κB in the nucleus and inhibits the transcriptional activity of antiapoptotic protein (Totzke et al. 2006). The structural basis of this inhibitory and activation mechanism was explained by docking studies (Manavalan et al. 2011), which have shown that IκBζ ARD associates with the DNA-binding domain of p50/p65 subunits of NF-κB and contains important residues that interact with the bases as well as sugar phosphate backbone present in the p50 and p65 subunit (Fig. 3). Hence, there will be no more p50/p65 subunits available to the promoter region, which ultimately results in inhibition of the transcription mediated by p50/p65 subunit. Generally, p65 subunits contain the TAD at the C-terminal end, which is important for its transcriptional activity (O'Shea and Perkins 2008). IκBζ inhibits p65 transactivation activity through its binding with the N-terminal DNA-binding domain.

Furthermore, IκBζ-p50/p50 complex revealed that ANK3-7 interact with the dimerization domain of the p50 subunit (Fig. 4). The binding orientation of IκBζ with this homodimer is similar to that of the classical IκBα-p50/p65 heterodimer. Although the binding orientation is the same, there might be some differences in the regulation of NF-κB-dependent gene expression by IκBα and IκBζ. Activation of p65-containing NF-κB heterodimer by LPS or IL-1 leads to the expression of NF-κB-dependent genes, including IκB proteins, IκBα and IκBζ. Following translocation, IκBα enters the nucleus, where it targets NF-κB p50/p65 dimers and removes them from DNA through the acidic PEST motif of IκBα and the basic DNA-containing surfaces of the NF-κB p65 subunit that likely disrupt protein/DNA binding. In contrast, IκBζ enters into the nucleus and targets the p50/p50 homodimer, which is already bound to the promoter region, thereby blocking the transcription due to the unavailability of the TAD. Overexpression experiments have suggested that IκBζ exhibited transactivation



IkBz, Fig. 4 IκBζ ARD-p50 homodimer interface. (**a**) The p50/p50 dimers are *blue* and *red* in the ribbon diagram. Docked IκBζ is *green* in the ribbon diagram. (**b**) The p50 (chain A)-IκBζ binding interface. Side chains of the amino acid contributing to the hydrogen bonding formation (marked as *black*) are represented by a stick model with the residue name and numbers shown next to them. (**c**) The p50 (chain B)-IκBζ binding interface is shown in a similar fashion as in (**b**)

potential (Motoyama et al. 2005); hence IκBζ mediates transcriptional activity by binding with DNA-bound p50/p50 homodimer, thereby providing a transactivation domain to the NF-κB complex. Such IκBζ-mediated transcription is important for the production of IL-6, antimicrobial peptides, lipocalin, hDB-2, and the genes listed in Table 1. Finally, it should be noted that, when compared with other IκB proteins, IκBζ possesses numerous functions that occur via binding with different nuclear proteins. Recent studies of MD (molecular dynamics) simulation of IκB have revealed that IκBζ possesses more thermodynamically flexible residues than other IκB members. These findings demonstrate that structural flexibility is the major factor that enables IκBζ to interact with different sets of nuclear proteins (Manavalan et al. 2010; Basith et al. 2013).

Summary

Studies of IκBζ have provided evidence for multistep regulation of inflammatory responses in TLR signaling. Upon cell activation by appropriate stimuli, primary responses are induced by rapid activation of the major transcription factor (NF-κB), which is activated through posttranslational modifications such as phosphorylation without de novo protein synthesis. During this period, transcriptional regulators such as IκBζ are induced via stimuli-specific mechanisms. Secondary response genes are activated, and primary responses are gradually diminished via the combinations of major transcription factors and inducible regulators. Since the genes that are activated via secondary responses also include other transcription factors, stimulus-specific transcriptional activation would proceed in a multistep fashion with time after the stimulation. In vitro studies have shown that these nuclear IκB proteins interact with the p50 or p52 subunits of NF-κB. Only p50/p52 double knockout mice, but not single knockout mice, exhibit severely defective immune disorders such as osteopetrosis. However, some immunological phenotypes occur in mice lacking only one nuclear IκB protein. This condition may be compensated by utilizing other IκB proteins. Further studies are required to clarify and discover new and detailed physiological aspects of the nuclear IκB proteins in the future by using mice devoid of two or all three nuclear IκB proteins. In conclusion, it can be seen that nuclear IκBζ not only contributes to NF-κB mediated transcription but also plays an important role in innate immune responses by modulating the expression of proinflammatory cytokines.

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IkB-Zeta

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