A Novel Member of the IκB Family, Human IκB-ζ, Inhibits Transactivation of p65 and Its DNA Binding*

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A novel member of the IκB family, human IκB-ζ, was identified by a differential screening approach of apoptosis-sensitive and -resistant tumor cells. The protein consists of 6 ankyrin repeats at its COOH terminus and shares about 30% identity with other IκB members. IκB-ζ associates with both the p65 and p50 subunit of NF-κB and inhibits the transcriptional activity as well as the DNA binding of the transcription factor. Interestingly, IκB-ζ is localized in the nucleus where it aggregates in matrix-associated deacetylase bodies, indicating that IκB-ζ regulates NF-κB activity rather than its nuclear translocation from the cytoplasm. IκB-ζ expression itself was regulated by NF-κB, suggesting that its activity is controlled in a negative feedback loop. Unlike classical IκB proteins, IκB-ζ was not degraded upon cell stimulation. Treatment with tumor necrosis factor-α, interleukin-1β, and lipopolysaccharide induced a strong induction of IκB-ζ transcripts. Expression of IκB-ζ was detected in different tissues including lung, liver, and in leukocytes but not in the brain. Suppression of endogenous IκB-ζ by RNA interference rendered cells more resistant to apoptosis, whereas overexpression of IκB-ζ was sufficient to induce cell death. Our results, therefore, suggest that IκB-ζ functions as an additional regulator of NF-κB activity and, hence, provides another control level for the activation of NF-κB-dependent target genes.

NF-κB is an evolutionarily conserved pleiotropic transcription factor that plays a crucial role in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis (Refs. 2–6; for review, see Ref. 1). The mammalian NF-κB/Rel family consists of RelA (p65), RelB, c-Rel, p50, and p52 that bind as homo- or heterodimers at κB sites in the DNA of their target genes. This combinatorial diversity contributes to the regulation of a distinct but overlapping set of genes, in that the individual dimers have distinct preferences for different κB sites that they can bind with distinguishable affinity and specificity. In addition, whether the transcription of a target gene is activated or repressed depends on the dimer combination. While RelA/p50, RelA/c-Rel, RelB/p50, and RelB/p52 heterodimers are transcriptional activators, p50/p50 and p52/p52 homodimers generally repress transcription (7–9). Regulation of the great diversity of genes requires a precise control of NF-κB, which is achieved by various mechanisms of posttranslational modification and subcellular compartmentalization as well as by interactions with other cofactors or corepressors. Dysregulation of NF-κB activation results in a wide range of human disorders including inflammatory and neurodegenerative diseases (10, 11) and different types of cancer (4, 12).

NF-κB activity is also tightly regulated by interaction with IκB proteins (13). Characteristic for all IκB proteins is a domain of multiple ankyrin repeats that bind to the conserved Rel homology domain of NF-κB proteins. Various members of the IκB family target different NF-κB complexes, e.g. IκB-α and IκB-β interact preferentially with p65/p50 and c-Rel/p50 heterodimers, whereas IκB-ε binds only to p65 and c-Rel hetero- and homodimers (14, 15), and Bcl-3 associates exclusively with p50 or p52 homodimers (16). Even if different IκB proteins interact with the same NF-κB dimers, the transcription factor can be regulated differentially due to functional differences between the IκB proteins. For instance, upon cell stimulation IκB-α is rapidly degraded, leading to an immediate but transient activation of NF-κB. In contrast, IκB-β persists over a longer time, and its degradation causes a more delayed and sustained activation of the transcription factor (14, 17).

In most cell types NF-κB is sequestered in the cytoplasm as an inactive complex bound to IκB proteins. A variety of stimuli, including tumor necrosis factor (TNF),5 interleukin-1 (IL-1), and lipopolysaccharide (LPS), lead to the phosphorylation of IκBα by the IκB kinase complex, which promotes their ubiquitination and proteasomal degradation (18). This event exposes a nuclear localization signal of NF-κB leading to its nuclear translocation and activation of target genes. The rapidly relocalized IκB-α, which itself is an NF-κB target gene, can enter the nucleus, dissociate NF-κB from the DNA, and translocate it back to the cytoplasm, thereby terminating gene transcription (19). In contrast to the typical IκB proteins (IκB-α, IκB-β, IκB-ε) that are preferentially localized in the cytoplasm and behave exclusively as inhibitors, Bcl-3 is a nuclear protein that preferentially promotes B-dependent gene transcription. Bcl-3 can cause derepression of transcription by removing p50 and p52 dimers, which are transcriptionally inactive, from κB sites (20). Alternatively, Bcl-3 can lead to direct transcriptional activation by forming a ternary complex with DNA through these homodimers (21–23).

In a continuous attempt to define molecular mechanisms involved in apoptosis, we performed a suppression subtractive hybridization approach with two HeLa cell lines that differ in their susceptibility to TNF (24, 25). Among several differentially expressed genes, a cDNA clone was identified encoding a novel IκB protein. In this study, we describe the structural and functional characterization of this protein, termed IκB-ζ. Unlike typical IκB proteins, IκB-ζ is not rapidly degraded but, rather, stably accumulates in the nucleus where it inhibits NF-κB

5 The abbreviations used are: TNF, tumor necrosis factor; IL-1, interleukin-1; LPS, lipopolysaccharide; siRNA, small interfering RNA; RT, reverse transcription; HEK cells, human embryonic kidney cells; PML, promyelocytic leukemia; GFP, green fluorescent protein; SMRT, silencing mediator of retinoid and thyroid hormone receptor; HDAC-5, histone deacetylase-5.

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activity. Interestingly, this inhibitory capability is opposite to the transactivating function of the nuclear Bcl-3 protein. Furthermore, IκB-ζ is only inducibly expressed in different cell types. Thus, these features of IκB-ζ, which are distinct from the cytosolic IκBs and Bcl-3, may confer an additional control mechanism of NF-κB activity.

MATERIALS AND METHODS

Reagents and Antibodies—Mo1eny murine leukemia virus reverse transcriptase, Lipofectamine/PLUS™ reagent, and the anti-c-Myc monoclonal antibody were from Invitrogen. Taq DNA polymerase and dNTPs were from Eppendorf (Hamburg, Germany), luciferin was from Applichem (Darmstadt, Germany), and o-nitrophenyl-β-D-galactopyranoside was from Calbiochem. TNF-α, LPS, anti-actin, and anti-FLAG antibodies, and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin were obtained from Sigma. IκB-α polyclonal antibody was purchased from Santa Cruz Biotechnol- ogy (Santa Cruz, CA), and anti-CD95 monoclonal antibody was from BioCheck (Münster, Germany). The mouse monoclonal antibody against human IκB-ζ was generated in our own laboratory. Hexamer nucleotides were from Roche Applied Science. Horseradish peroxidase-labeled goat anti-mouse IgG and anti-rabbit IgG were from Bio-Rad, RNAguard and protein G-Sepharose were from Amersham Bio- sciences, IL-1β, BAY 11-7082, and MG-132 were from Biomol (Ham- burg, Germany), and Mitotracker Red was from Molecular Probes (Lei- den, The Netherlands). The pSilencer small interfering RNA (siRNA) expression vector kit was from Ambion (Huntingdon, Cambridgeshire, UK), and the Human RNA Master and MTN Blot were from Clontech (Palo Alto, CA).

Cell Culture and Treatment—The human cervix carcinoma cell lines HeLa H21 and D98 (24), MCF-7/casp-3 human breast carcinoma cells (26), human monocytic MonoMac6 cells, and HT-1080 human fibro- sarcoma cells were cultured in RPMI 1640 medium. A549 human lung carcinoma cells were maintained in Ham’s F-12, and HepG2 human hepatocellular carcinoma cells, U2OS human osteosarcoma cells, 293 carcinoma cells were maintained in Ham’s F-12 medium. A549 human lung carcinoma cells were cultured in RPMI 1640 medium. A549 human lung carcinoma cells were cultured in RPMI 1640 medium. A549 human lung carcinoma cells were cultured in RPMI 1640 medium.

Northern and Dot Blot Hybridization—To detect expression of IκB-ζ in normal human tissues, the commercially available MTN and RNA Master Blots (Clontech) were used. A 714-bp BamHI/Hind III fragment was taken as probe. Hybridization with the α-32P-labeled cDNA probe and washing procedure was carried out according to standard protocols.

Reverse Transcription (RT)-PCR—For semiquantitative mRNA expression studies HeLa D98 and MCF-7/casp-3 cells were exposed to TNF and IL-1β, and MonoMac6 cells were exposed to LPS for the indicated times. To investigate a possible IκB-ζ regulation by NF-κB, HeLa D98 cells were pretreated with BAY 11-7082 or MG-132 for 1 h before stimulation with IL-1. The 5′ (5′-GCTAATCCCCATGCAGACCTT-3′) and 3′ (5′-GAACGTGTCACCATCTGCAT-3′) primers for the amplification of the IκB-ζ DNA fragment and the 5′ (5′-AAAGATCCGCACATTTCC- CAGGGCGCCGAGAG-3′) and 3′ (5′-TTTAAGCTTTAAGTCAGA CGCTGCGCTCC-3′) primers for amplification of the IκB-α DNA were synthesized commercially (Invitrogen). For standardization purposes each RT sample was subjected to PCR for glyceraldehyde-3-phosphate dehydrogenase (5′ primer, 5′-GCCAAAGGGTTCATCATCTCT-3′; 3′ primer, 5′- GTAGAGGCAGGGATGATGTTC-3′) using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Light emission was measured after injection of 100 μl of luciferin (0.3 mg/ml), and the values were normalized to β-galactosidase activity.

Luciferase Reporter Gene Assay—To determine NF-κB-dependent transcriptional activity, a NF-κB luciferase reporter plasmid containing six κB binding sites in front of the luciferase promoter was used. A β-galactosidase (lacZ) vector served as an internal control for transfection efficiency. HEK 293 cells were seeded into 6-well plates 1 day before transfection. The cells were transiently transfected with the NF-κB luciferase plasmid and the lacZ plasmid together with the indicated expression plasmids using the Lipofectamine/PLUS reagent. 24–48 h after transfection, cells were either left unstimulated or were stimulated with TNF or IL-1 for 6 h, lysed in 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1 mM dithiothreitol, 2 mM ATP) using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Light emission was measured after injection of 100 μl of luciferin (0.3 mg/ml), and the values were normalized to β-galactosidase activity.

Electrophoretic Mobility Shift Assay—HEK 293 cells were plated at 3 × 105/well in 6-well plates 1 day before transfection with 500 ng of the p65 or p50 plasmid and increasing amounts (1–16 μg) of the IκB-ζ or, as a control, the IκB-α plasmid. The total amount of transfected DNA was normalized with the empty vector control. Cells were lysed 48 h after transfection, and the binding reaction was performed for 30 min at room temperature in a 20-μl volume containing 4 μl of extract, 4 μl of 5× binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol, 20% Ficoll), 2 μg of poly(dI-dC), 2 μg of bovine serum albumin, and the 32P-labeled NF-κB oligonucleotide. Samples were loaded on a 4% non-denaturing polyacrylamide gel and run in 0.5× Tris borate EDTA buffer, pH 8. After drying, the gels were exposed to x-ray films.
Preparation of Whole, Cytosolic, and Nuclear Cell Extracts—To obtain whole protein extracts, cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM dithiothreitol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml concentrations each of aprotinin, leupeptin, and pepstatin for 30 min on ice and then centrifuged at 13,000 rpm for 30 min at 4 °C. For the preparation of cytosolic and nuclear extracts, HeLa D98 cells were collected by centrifugation and broken by 4 freeze-thaw cycles in 10 mM HEPES, pH 7.9, 5 mM MgCl₂, 0.25 M sucrose, 5 mM NaF, 10 mM -mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. After incubation for 10 min on ice and a centrifugation step (14,000 rpm, 5 min, 4 °C), the supernatant (cytosolic fraction) was transferred to a fresh tube. The pellet was resuspended in high salt buffer (400 mM NaCl, 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.1 mM EDTA, 25% glycerol, 5 mM NaF, 10 mM -mercaptoethanol) containing protease inhibitors and incubated for 30 min on ice. Thereafter, the freeze-thaw cycle was repeated four times, and the nuclear extract (supernatant, soluble fraction) as well as the nuclear pellet (insoluble fraction) was collected by centrifugation. Protein concentrations were determined with the Bio-Rad protein assay.

SDS-PAGE and Western Blotting—Equal amounts of the proteins were separated in a 10% SDS-polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked with 5% bovine serum albumin in phosphate-buffered saline, and incubated overnight at 4 °C with the IκB-α, IκB-β, poly(ADP-ribose) polymerase, caspase-3, or actin antibodies in blocking buffer. After incubation for 1 h with the secondary antibody (horseradish peroxidase-labeled goat anti-mouse IgG or anti-rabbit IgG) in phosphate-buffered saline, 0.2% Tween, 5% bovine serum albumin, the proteins were visualized by enhanced chemiluminescent staining using ECL reagents (Amersham Biosciences).

Immunoprecipitation—HEK 293 cells were transiently transfected with FLAG-tagged IκB-α or IκB-β together with Myc-tagged p65 or p50 for 24 h using Lipofectamine/PLUSTM reagent. Then lysis was performed for 30 min on ice in 20 mM HEPES, pH 7.4, 84 mM KCl, 10 mM MgCl₂, 0.2 mM EDTA, 0.2 mM EGTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. After centrifugation the supernatants were incubated with protein G-Sepharose and either anti-FLAG or anti-IκB protein antibody overnight at 4 °C. The immunoprecipitates were resolved by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, blocked, and incubated overnight at 4 °C with a monoclonal anti-c-Myc antibody.
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(Invitrogen, 1:5000). A horseradish peroxidase-labeled goat anti-mouse IgG served as a secondary antibody.

**Immunofluorescence Microscopy**—Cells were seeded on coverslips and transfected with the indicated plasmids. FLAG-tagged silencing mediator of retinoid and thyroid hormone receptor (SMRT) and yellow fluorescent protein-tagged histone deacetylase-5 (HDAC-5) constructs were obtained from Dr. R. M. Evans. After 18 h of incubation, the cells were fixed for 5 min with 3.7% formaldehyde in phosphate-buffered saline and incubated in IF buffer (4% bovine serum albumin, 0.05% saponin in phosphate-buffered saline). Cells were then labeled with primary antibodies (anti-PML, Santa Cruz Biotechnology; anti-FLAG, Sigma) in IF buffer at 4 °C overnight. After washing, incubation with secondary antibodies (Alexa-594-coupled chicken anti-mouse IgG, Molecular Probes) was performed for 4 h. Finally, coverslips were mounted on glass slides using DAKO fluorescence mounting medium supplemented with Hoechst 33342 (500 ng/ml). Digital pictures were captured using a Leica TCS-SP2/AOBS confocal microscope.

**Construction of Small Interfering RNAs and Stable Transfection**—For the suppression of IκB-ζ, two different siRNAs were designed using the Dharmacon siDESIGN Center. The selected sense sequences were 5’-GATTGCTTGTCTGATGGAC-3’ and 5’-TACTCGGAACCTTG-GAGAC-3’. Oligonucleotides containing the sense, loop, antisense sequence, and a polythymidine tract were annealed and ligated into the pSilencer siRNA expression vector according to the manufacturer’s instructions (Ambion). HeLa D98 cells were stably transfected by electroporation using the Bio-Rad gene pulser (500 microfarads, 160 V). After hygromycin selection, several clones were obtained, and the successful reduction of IκB-ζ expression was controlled by Western blot analysis.

**Cytotoxicity Assay**—Parental HeLa D98 cells and three different clones stably expressing IκB-ζ siRNA were seeded into 96-well microtiter plates at a density of 3–4 × 10^4/ml. Before exposure to various concentrations of the apoptosis-inducing reagents TNF and anti-CD95, each combined with cycloheximide (10 μg/ml), the cells were stimulated with IL-1 for 2 h. For determination of cell death, the standard crystal violet assay was employed (31). The viable cells were stained with 20% methanol containing 0.5% crystal violet and solubilized in 33% acetic acid. The absorbance was measured at A_{560} nm. Percentage-specific cell death is defined as 100 – (A_{560} of test well × 100/A_{560} of untreated well). Each experiment was performed independently at least three times, and an individual experiment was carried out in triplicate.

**RESULTS**

**Isolation of a cDNA Encoding a Novel Member of the IκB Family**—To identify genes involved in the modulation of apoptosis, the human HeLa cell lines D98 (TNF-sensitive) and H21 (TNF-resistant) were exposed to TNF combined with cycloheximide for 1 h, and the mRNAs were subjected to the suppression subtractive hybridization procedure. Among several differentially expressed genes, a cDNA clone was identified encoding a protein that contains six ankyrin repeats at its COOH terminus sharing about 30% identity with different members of the IκB protein family. Northern blot analysis revealed that the mRNA has a length of about 3.5 kilobases kb (data not shown). The full-length cDNA contains an open reading frame of 1857 bp encoding a protein of 618 amino acids. The sequence data were deposited in the data base (GenBank™ DQ224339). The nucleotide and amino acid sequences are shown in Fig. 1, A and B, respectively. Besides the COOH-terminal ankyrin repeats, the gene product consists of a potential PEST domain which, however, in contrast to IκB-α and IκB-β, is located at the NH₂ terminus (amino acids 84–104). The other NH₂-terminal regions exhibit no significant homology with other proteins.

The structural similarity to IκB members suggested that we had identified a novel IκB protein. Data base analysis revealed an 80% homology to a mouse protein, termed molecule possessing ankyrin repeats induced by lipopolysaccharide (MAIL). This protein exists in two isoforms, MAIL-L and MAIL-S, which is identical to IκB-ζ (32, 33). During the course of our functional characterization of the novel gene product, sequences of different human MAIL isoforms were deposited in the data base. Our identified gene product is most similar to MAIL-S (GenBank™ AF548362), with the difference that it lacks exon 2 (nucleotides 193–268) in the 5’-untranslated region and therefore represents a to date unknown isoform of MAIL. Because the MAIL proteins are also known as nuclear factor of κ light polypeptide gene enhancer in B-cells
Inhibitor ζ (NFKBIZ), transcript variant 1 and transcript variant 2, we termed the novel isoform human IκB-ζ, transcript variant 3. In the following this variant is referred to as IκB-ζ.

**IκB-ζ is Expressed in Different Human Tissues**—To obtain an mRNA expression profile of IκB-ζ, a commercially available RNA dot blot was used. Expression was detectable in different human tissues such as lung, placenta, and liver and also in peripheral blood leukocytes. In contrast, IκB-ζ was not expressed in different regions of the brain (Fig. 1C). To verify these results, a Northern blot was performed with mRNA from 12 different human tissues. Again, lung and peripheral blood leukocytes showed the highest expression, liver and placenta showed moderate expression, and spleen, kidney, skeletal muscle, and heart showed a low expression level, whereas no expression could be detected in the brain (Fig. 1D).

**NF-κB Promoter Activity Is Inhibited by the COOH-terminal Domain of IκB-ζ**—The presence of ankyrin repeats in the COOH-terminal region of IκB-ζ suggested that we had identified a bona fide NF-κB inhibitor, which encouraged us to investigate its effect on the regulation of NF-κB activity. To this end, HEK 293 cells were transiently transfected with a κB-dependent luciferase reporter plasmid together with a β-galactosidase vector and a plasmid containing the cDNA for either IκB-ζ or as a control IκB-α. As measured in a luciferase reporter gene assay, activation of NF-κB was observed when cells were exposed to TNF (Fig. 2A). IκB-ζ was able to inhibit NF-κB activity in TNF-stimulated as well as in unstimulated cells in a concentration-dependent manner, indicating that IκB-ζ indeed belongs to the class of proteins that inhibit NF-κB. Compared with TNF, the cytokine IL-1 was a weaker NF-κB activator in HEK 293 cells. Nevertheless, the IL-1-induced NF-κB activation was also reduced by IκB-ζ (Fig. 2B), confirming its inhibitory effect on NF-κB. To find out which functional domain of IκB-ζ is responsible for this inhibition, truncated mutants of IκB-ζ were constructed. The COOH-terminal region containing the ankyrin repeats efficiently inhibited NF-κB activity to an even greater extent than full-length IκB-ζ, whereas the NH₂-terminal domain had no effect (Fig. 2C). As a control, in all experiments the effect of IκB-α was investigated in parallel. IκB-α was a much stronger NF-κB inhibitor, indicating that the transcriptional activity of NF-κB is regulated differentially by these two IκB proteins. Such a weaker inhibition of NF-κB activity in comparison to IκB-α has been also reported for IκB-ε (34).

**IκB-ζ Binds to the p65 and p50 Subunits of NF-κB and Inhibits Its DNA Binding and Transactivation Activity**—Because IκB-ζ was identified as an NF-κB inhibitor, the question arose of which NF-κB subunits associate with IκB-ζ. Therefore, HEK 293 cells were transiently transfected with p65 or p65/p50 and IκB-ζ or IκB-α together with a β-galactosidase-dependent luciferase reporter and a β-galactosidase plasmid for 48 h. NF-κB activity was measured in relative light units (RLU) and normalized to β-galactosidase activity.

Next, we investigated whether the binding of IκB-ζ to p65 affected its transactivation activity. Ectopic expression of p65 caused a strong activation of NF-κB that was substantially inhibited by IκB-ζ and almost completely blocked by IκB-α (Fig. 3C). Interestingly, although both IκB-α and IκB-ζ were capable of inhibiting NF-κB activity, IκB-ζ showed a stronger effect than IκB-α, suggesting that IκB-ζ might influence the binding activity of NF-κB to the DNA. Indeed, gel-shift analyses revealed that IκB-ζ inhibited the DNA binding of p65/p50, since the p65 as well as the p50 band disappeared gradually with increasing amounts of IκB-ζ (Fig. 3B). Compared with IκB-α, a higher amount of IκB-ζ was necessary to obtain the same effect, demonstrating that IκB-α functions as a much more efficient inhibitor of NF-κB DNA binding than IκB-ζ.
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proteins associated with the same NF-κB subunits, they differentially regulated DNA binding activity of NF-κB and transactivation of p65.

IκB-ζ is Inducible at the mRNA and Protein Level—Further characterization of IκB-ζ, its regulation was investigated. HeLa D98 cells were exposed to TNF and IL-1, and the mRNA expression was analyzed in comparison to IκB-α. Very low levels of IκB-ζ mRNA were observed in unstimulated cells (Fig. 4A). TNF and IL-1 were able to induce IκB-ζ mRNA in a time-dependent manner. Maximal expression of the IκB-ζ mRNA occurred within 30–60 min after stimulation. Although a slight decline was observed afterward, a relatively high expression level persisted over the whole time course of the experiment. A similar induction profile was detected for transcription of IκB-α (Fig. 4A). The stimulatory effect of the different agents was not restricted to the HeLa cells, as MCF-7 breast cancer cells were similarly affected (Fig. 4B). Because the IL-1 and the LPS receptor activate similar intracellular signaling pathways, it could be assumed that human IκB-ζ is also inducible by LPS. Because neither the HeLa cells nor the MCF-7 cells responded toward LPS, the human monocytic cell line MonoMac6 was treated with LPS. In fact, also LPS led to an increased IκB-ζ expression with a time course similar to that observed for TNF and IL-1 (Fig. 4C).

In the next set of experiments we examined the regulation of IκB-ζ protein expression using a monoclonal antibody that we had generated against the recombinant protein. In quiescent MCF-7 cells IκB-ζ was not expressed, but after stimulation with IL-1 a maximal induction of IκB-ζ protein expression occurred within 1–2 h and declined thereafter (Fig. 5A, left panel). In contrast, preexisting IκB-α was degraded rapidly within 15–30 min and resynthesized 1 h after cytokine stimulation. IL-1-stimulated IκB-ζ expression showed a similar induction pattern in HeLa cells (Fig. 5A, middle panel). A weaker but clearly significant induction of IκB-ζ protein was also seen after stimulation of HeLa cells with TNF (Fig. 5A, right panel). Furthermore, a rapid induction of IκB-ζ protein was found in IL-1-stimulated cell types, such as the human lung carcinoma cell line A549, the hepatocellular carcinoma cell line HepG2, and human HaCaT keratinocytes (Fig. 5B).

As for the mRNA expression studies, MonoMac6 cells were used to examine the effect of LPS on IκB-ζ protein expression. Indeed, the IκB-ζ protein level increased dramatically starting 1 h after exposure to LPS and persisted over at least 24 h (Fig. 5C). It is noteworthy that in LPS-stimulated monocytes we reproducibly observed a biphasic induction of IκB-ζ protein expression. Furthermore, in addition to the 85-kDa band of IκB-ζ, in these cells a protein of ~70 kDa was induced that presumably corresponds to the short isoform of IκB-ζ.

IκB-ζ is Regulated by NF-κB—After stimulation of NF-κB, both the mRNA of IκB-ζ and IκB-α were up-regulated. Therefore, we asked whether this transcription factor regulates IκB-ζ in a similar manner as known for IκB-α (35, 36). To this end, HeLa D98 cells were incubated for 1 h before the addition of IL-1 with the proteasome inhibitor MG-132, which prevents the degradation of IκB-α and, thus, NF-κB activation. Analysis of protein expression revealed that, as expected, IL-1 treatment induced a strong degradation of IκB-α that was almost completely prevented in the presence of MG-132 (Fig. 6). Interestingly, the IL-1-induced IκB-ζ expression was markedly reduced by MG-132, indicating that NF-κB activation might be necessary for the induction of IκB-ζ (Fig. 6, upper left panel). This assumption was supported at the transcriptional level, since the IL-1-induced mRNA expression levels of both, IκB-α and IκB-ζ, decreased when cells were pretreated with MG-132 (Fig. 6, upper right panel). These results were further confirmed by using the IκB kinase inhibitor BAY 11-7082 that prevents the phosphorylation of IκB-α and, hence, NF-κB activation. IL-1-induced IκB-ζ expression was diminished dose-dependently at the mRNA and protein level by BAY 11-7082 (Fig. 6, lower panels). Thus, these data show that IκB-ζ is regulated, at least in part, by NF-κB.

IκB-ζ Localizes to Intracellular Matrix-associated Deacetylase Bodies—To determine the intracellular localization of IκB-ζ, cytoplasmic and nuclear fractions were isolated from HeLa D98 cells and analyzed by Western blotting. IκB-ζ was preferentially expressed in the nuclear fractions (Fig. 7A). Under basal conditions a very low expression of IκB-ζ was detected that was strongly induced after a 2–4 h treatment with IL-1 (Fig. 7A). As a control for the purity of the subcellular fractions, the membrane was rebotted with anti-poly(ADP-ribose) polymerase and anti-caspase-3 antibodies as nuclear and cytoplasmic markers, respectively. Additionally, the localization was examined by immunofluorescence microscopy in cells transiently transfected with GFP-tagged
Interestingly, these analyses revealed that \( \text{IKB-}\xi \) was not evenly distributed in the nucleus but aggregated in distinct dot-like structures (Fig. 7B).

To identify the nature of these nuclear bodies, we performed detailed colocalization studies using different marker proteins. \( \text{IKB-}\xi \) neither colocalized with promyelocytic leukemia (PML) protein (Fig. 8, right panel) nor with SC-35 (data not shown), suggesting that it was not associated with PML or RNA splicing bodies. However, the localization of \( \text{IKB-}\xi \) completely coincided with the nuclear corepressor SMRT (Fig. 8, left panel), which has been found to be retained in the recently identified matrix-associated deacetylase bodies (37). Indeed, also HDAC5 was localized in same subnuclear structures (Fig. 8, middle panel). The results, therefore, suggest that \( \text{IKB-}\xi \) is a nuclear I\( \kappa \)B protein that might function through modulating HDAC activity.

\( \text{IKB-}\xi \) Rendes Cells More Sensitive to Cell Death—Because we had originally identified \( \text{IKB-}\xi \) as a TNF-inducible gene product that was overexpressed in apoptosis-sensitive but not in apoptosis-resistant HeLa cells, the functional role of \( \text{IKB-}\xi \) in the regulation of cell death was examined. RNA interference was used to suppress the IL-1-induced expression of \( \text{IKB-}\xi \). HeLa D98 cells were stably transfected with the pSilencer vector containing different siRNAs for \( \text{IKB-}\xi \). Western blot analysis confirmed that, compared with the wild-type cells, the IL-1-induced \( \text{IKB-}\xi \) protein expression was strongly or even almost completely inhibited in the hygromycin-selected siRNA clones (Fig. 9A). The \( \text{IKB-}\xi \) knock-down and wild-type cells were then either left unstimulated or pretreated with IL-1 for 2 h before the exposure to various concent...
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FIGURE 9. IκB-ζ promotes apoptosis. A and B, RNA interference-mediated knockdown of endogenous IκB-ζ renders cells resistant to death receptor-mediated apoptosis. A, Western blot analyses for the status of IκB-ζ expression in HeLa D98 cells that were stably transfected with siRNA-1 (clone 1 and 2) or siRNA-2 (clone 3). IL-1-induced IκB-ζ expression in parental cells (wt) is shown as well as the down-regulation of IκB-ζ in the IL-1-treated clones. Cells were either left untreated or treated with IL-1 for 2 h, and the lysates were subjected to Western blot analysis. B, cell death assessment of wild type (wt) and siRNA cells (clones 1–3) that were treated with IL-1 for 2 h before exposure to the indicated concentrations of TNF or anti-CD95 for 7 and 5 h, respectively. C, overexpression of IκB-ζ is sufficient to induce cell death. Phase contrast and fluorescent micrographs of HT-1080 cells after 48 h of infection with a retrovirus encoding bicistronic mRNAs for the GFP control (left panels), GFP and IκB-ζ (middle panels), or GFP and IκB-α (right panels). The Western blot analysis on the right shows the expression status of the two IκB proteins and actin after infection with the indicated retroviruses. The results demonstrate that, in comparison to the GFP control, both IκB-α and IκB-ζ exert a cytotoxic effect, as indicated by the condensation and detachment of the GFP-positive cells.

DISCUSSION

Using the suppression subtractive hybridization technique, we have identified a gene product that was differentially expressed in apoptosis-sensitive and -resistant HeLa cells. The gene encodes a novel IκB protein with six ankyrin repeats and a potential NH₂-terminal PEST domain. Sequence comparison revealed that it is most similar to human MAIL-S (GenBank™ AF548362) or nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor ζ (NFκBIZ), transcript variant 2 (GenBank™ NM_001005474), which has, however, not been characterized so far. Based on this information, we termed our newly identified clone IκB-ζ, transcript variant 3. Although both MAIL-S, which had been deposited in the data base during this study, and our gene product encode a protein of 618 amino acids, the lack of exon 2 in the 5’-untranslated region of IκB-ζ may have regulatory consequences.

IκB proteins interact via their ankyrin repeats with the Rel homology domain of NF-κB. The transcriptional activity of NF-κB was inhibited by full-length IκB-ζ and even more by a COOH-terminal construct containing the ankyrin domain. Thus, it seems that elements in the amino terminus can influence the effect of IκB-ζ directly or that the structure of the carboxyl terminus is more favorable to interact with NF-κB subunits.

Control of NF-κB activation is partially regulated by preferential association of specific NF-κB dimers with certain members of the IκB family. IκB-α and IκB-β interact with p65/p50 and c-Rel/p50 heterodimers, IκB-ε associates only with homo- or heterodimeric complexes containing p65 and c-Rel (14, 15, 34), and Bcl-3 interacts specifically with p50 and p52 homodimers (22). In contrast, p100 and p105, which are precursors of the Rel proteins p52 and p50, respectively, appear to bind efficiently to all Rel proteins. Moreover, functional differences between various IκB proteins allow the binding to the same NF-κB dimer. For example, the classical NF-κB heterodimer p65/p50...
interacts with IκB-α and IκB-β, but both IκB proteins show differences in their response to various NF-κB inducers and in their kinetics of degradation (14, 38). In our study we were able to demonstrate that IκB-ζ also associates with p65 and p50 but was less effective in inhibiting NF-κB transcriptional activity as compared with IκB-α. In addition, a higher amount of IκB-ζ was necessary to inhibit the binding of NF-κB to the DNA, possibly due to a higher affinity of p65/p50 to IκB-α. A considerable weaker inhibition of NF-κB DNA binding compared with IκB-α has been reported for IκB-ε (34).

Unlike the classical IκB proteins, our study shows that the novel IκB-ζ protein is not controlled by inducible degradation. Furthermore, IκB-ζ was not expressed in quiescent cells but was strongly induced upon stimulation, suggesting that different mechanisms take place in the regulation of NF-κB by these inhibitors. Because IκB-ζ is localized in the nucleus, it is likely that it controls the activated NF-κB in this compartment rather than the translocation of NF-κB into the nucleus. Nevertheless, the remarkable up-regulation of IκB-ζ after NF-κB activation and its down-regulation by BAY and MG-132 together with the identification of IκB-ζ as NF-κB inhibitor suggest that this IκB protein is regulated by NF-κB in a negative feedback loop. Negative autoregulatory loops provide an effective mechanism for controlling NF-κB activity, especially because fast and slow negative feedback loops exist. In contrast to the rapid IκB-α expression (39), IκB-ε represents a slower negative feedback regulatory mechanism (15). Because the mRNAs of IκB-ζ and IκB-α were induced with similar kinetics, our data suggest that IκB-ζ represents an additional fast negative feedback loop for the control of NF-κB activation.

Our results indicate that there might exist species-specific differences in the regulation of NF-κB by IκB-ζ. Although human and mouse IκB-ζ (also called MAILS-5 or I1NAP) are highly homologous at their COOH terminus, both proteins differ particularly at their NH2 terminus. The mouse homologue has been demonstrated to interact only with the p50 subunit of NF-κB but not with p65 (33). Another study (40) even found no interaction of mouse IκB-ζ with p50, p52, or p65. Therefore, human IκB-ζ cannot only regulate the DNA binding of NF-κB but also its transcriptional activity through association with the NF-κB subunit p65. Moreover, a different gene expression profile might occur in human and mouse systems due to the different composition of the NF-κB dimers.

Consistent with our data, mouse IκB-ζ is induced in response to LPS and IL-1 (33). The cytoplasmic domains of the Toll-like receptors and the IL-1 receptor are homologous and, hence, activate the same signaling pathways leading to the activation of NF-κB (41, 42), which then can induce IκB-ζ in an autoregulatory manner. It was recently shown that induction of mouse IκB-ζ through Toll-like/IL-1 receptors was specifically due to mRNA stabilization (43). In contrast, our data also demonstrate that the induction of human IκB-ζ is exerted additionally via the TNF receptor pathway. Whereas TNF had no effect on the induction of mouse IκB-ζ (33), the human homologue was TNF-responsive. Whether the differential inducibility of human and mouse IκB-ζ reflects a species- or a cell type-specific difference remains to be shown. In this context, it has been reported that NF-κB activity was required but not sufficient for the induction of mouse IκB-ζ (44). Our data also suggest that human IκB-ζ is regulated by NF-κB. Whether NF-κB activity is sufficient to induce human IκB-ζ is under further investigation.

Besides its function as a negative regulator of NF-κB activation, it was recently demonstrated that mouse IκB-ζ exerts transcriptional activity. Analyses of IκB-ζ-deficient mice revealed that it was essential for the expression of numerous LPS-inducible genes including IL-12 p40, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte-CSF), C/EBP-δ, and endothelin (45). Moreover, the Toll-like receptor/IL-1 receptor-mediated production of IL-6 was profoundly inhibited in IκB-ζ-deficient cells. In this context it has been reported that the IL-6 promoter was activated by mouse IκB-ζ in the macrophage cell line RAW 264.7. Interestingly, whereas IκB-ζ enhanced LPS-mediated IL-6 promoter activity (45), IκBNS, another related nuclear IκB protein, suppressed IL-6 gene transcription (46). Transcriptional activation of NF-κB target genes has been also shown for Bcl-3 that like IκB-ζ is not degraded by the proteasome and also localized in the nucleus. Bcl-3 can cause DNA-bound p50 homodimers to dissociate from the κB site, permitting these inhibiting NF-κB species to be replaced by the transactivating members p65, RelB, or c-Rel. Bcl-3 can also form a ternary complex with DNA-bound p50 or p52 homodimers and activate transcription directly, an activity that requires both NH2- and COOH-terminal domains of Bcl-3 (21, 22, 47). To date, only the cyclin D1 promoter has been shown to be directly activated by Bcl-3 p52 complexes (48, 49). A possible function of human IκB-ζ as a transcriptional activator has not been investigated so far. However, recently it has been suggested that the human MAIL isoforms might harbor a transactivation domain in their NH2 terminus (50). Therefore, it is conceivable that human IκB-ζ may directly activate the transcription of target genes. Nevertheless, if so, the subset of target genes affected and the molecular mechanism for their induction might be different from mouse IκB-ζ due to the TNF-inducibility of human IκB-ζ and its association with the p65/p50 heterodimer.

In line with its function as a negative regulator of NF-κB, we found a proapoptotic effect of human IκB-ζ. Suppression of endogenous IκB-ζ rendered cells more resistant to apoptosis induced by TNF and anti-CD95, whereas overexpression of IκB-ζ was sufficient to induce cell death. Although the molecular mechanisms of modulation of cell survival by IκB-ζ are currently unknown, these results are consistent with the anti-apoptotic function of NF-κB. Products of NF-κB target genes that inhibit apoptosis include members of the Bcl-2 family (Bcl-xL, Bcl-1/A1), inhibitors of apoptosis proteins (c-IAP1, c-IAP2, XIAP), adaptor molecules (TNF receptor-associated factor (TRAF1), TRAF2), and FLICE inhibitory protein (c-FLIP) (for review, see Ref. 5). It has become increasingly clear that the activation of NF-κB is not only controlled in the cytoplasm but, presumably even more importantly, also modulated in the nucleus. Not only phosphorylation and acetylation of NF-κB itself play a critical role in the regulation of its transcriptional activity (51, 52) but also interactions with other nuclear proteins including histone deacetylases, coactivators, and other transcription factors (9, 53–56). Our finding that IκB-ζ colocalizes with HDAC5 and the corepressor SMRT in matrix-associated deacetylase nuclear bodies is, therefore, highly intriguing. Although further functional studies in this respect are required, the localization of IκB-ζ in these subnuclear structures points to role of IκB-ζ in modulation of HDAC activity and chromatin structure. Our study together with the results of other groups, thus, suggests that nuclear IκB proteins add an additional layer to the already complex regulatory mechanisms that activate or repress NF-κB targets genes. Although the molecular mechanism of NF-κB regulation by human IκB-ζ has to be elucidated, this IκB protein might play an important role as regulator of NF-κB activity, especially because it is, to our knowledge, the first nuclear IκB protein that binds to the p65 and p50 subunit of NF-κB and regulates the transcription factor in a negative way.

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