IκBε provides negative feedback to control NF-κB oscillations, signaling dynamics, and inflammatory gene expression

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NF-κB signaling is known to be critically regulated by the NF-κB–inducible inhibitor protein IκBα. The resulting negative feedback has been shown to produce a propensity for oscillations in NF-κB activity. We report integrated experimental and computational studies that demonstrate that another IκB isoform, IκBε, also provides negative feedback on NF-κB activity, but with distinct functional consequences. Upon stimulation, NF-κB–induced transcription of IκBε is delayed, relative to that of IκBα, rendering the two negative feedback loops to be in antiphase. As a result, IκBε has a role in dampening IκBα-mediated oscillations during long-lasting NF-κB activity. Furthermore, we demonstrate the requirement of both of these distinct negative feedback regulators for the termination of NF-κB activity and NF-κB–mediated gene expression in response to transient stimulation. Our findings extend the capabilities of a computational model of IκB–NF-κB signaling and reveal a novel regulatory module of two antiphase negative feedback loops that allows for the fine-tuning of the dynamics of a mammalian signaling pathway.

Introduction

The NF-κB family of transcription factors controls diverse mammalian signaling responses that mediate cell survival, inflammation, and immune response (Gerondakis et al., 1999; Li and Verma, 2002; Hoffmann and Baltimore, 2006). Functional NF-κB exists in a dimeric form that is composed of combinations of five proteins containing a Rel homology region, i.e., cRel, RelA, RelB, p50, and p52 (Ghosh et al., 1998; Hoffmann and Baltimore, 2006). In resting cells, the NF-κB dimer is bound to inhibitor IκB proteins, i.e., IκBα, -β, and -ε, which inhibit NF-κB DNA-binding activity and prevent its nuclear accumulation. Activation of the NF-κB signaling pathway relies upon signal-dependent phosphorylation and degradation of the IκB proteins that result in subsequent nuclear translocation of the NF-κB dimer (Ghosh et al., 1998).

Termination of NF-κB activity after cellular stimulation is critical, as deregulated inflammatory gene expression can be detrimental to the health of the organism, and several attenuation mechanisms have been described (Greten and Karin, 2004; Li et al., 2005). Importantly, IκBα, which is a target gene of NF-κB, is induced by numerous NF-κB–inducing stimuli, resulting in the termination of NF-κB DNA-binding activity and nuclear localization (Scott et al., 1993; Ghosh et al., 1998).

Temporal control of NF-κB activity has been shown to mediate stimulus-specific gene expression programs in response to different inflammatory stimuli (Werner et al., 2005), and understanding the dynamic regulation of NF-κB by IκB proteins is of critical importance. Negative feedback that is mediated by IκBα was shown to confer the propensity for oscillatory NF-κB nuclear activity, both when examined biochemically in gene knockout cells containing only the IκBα isoform (Fig. 1 B; Hoffmann et al., 2002) and when examined by microscopy using transiently transfected IκBα and RelA proteins fused to fluorescent moieties (Fig. 1 D; Nelson et al., 2004). The oscillations are not apparent in cells containing all three IκB proteins at normal expression levels (Fig. 1, A and C; Hoffmann et al., 2002; Barken et al., 2005).

These observations suggested that IκBβ and/or -ε proteins play a role in dampening IκBα-mediated oscillations and determining the dynamics of NF-κB activity. The mechanism that confers dampening of oscillations in NF-κB activity was proposed to involve the nuclear accumulation of newly synthesized IκBβ that binds nuclear and promoter-bound NF-κB and

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Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; MEF, murine embryonic fibroblast; RPA, RNase protection assay.

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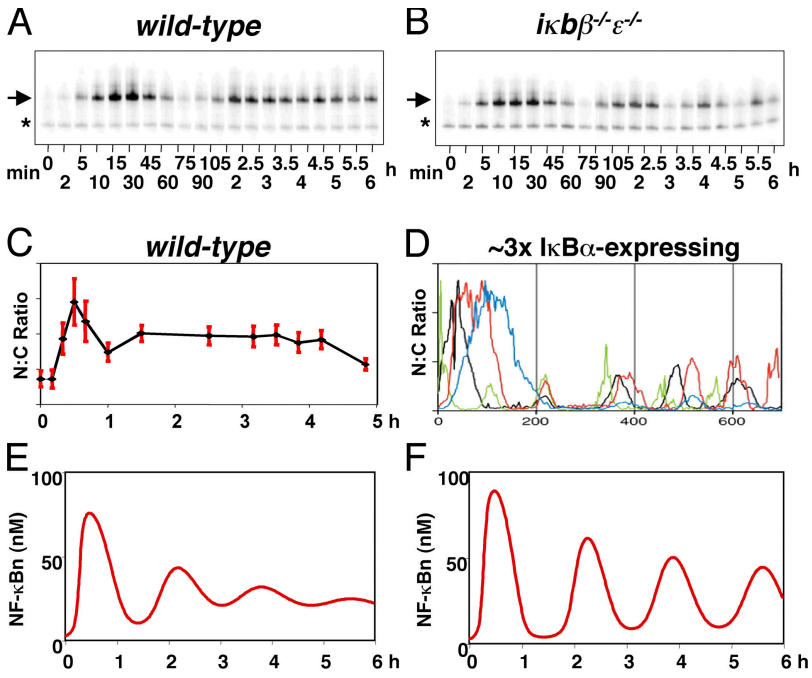

Figure 1. **Oscillations in NF-κB nuclear localization.** EMSAs show nuclear localization of NF-κB in wild-type (A) and $i\kappa b\beta^{-/-}\varepsilon^{-/-}$ MEF cells (B) stimulated with TNF (adapted from Hoffmann et al., 2002). Arrows indicate specific nuclear NF-κB–binding activity. Asterisks indicate nonspecific DNA-binding complexes. (C) Quantitation of immunohistochemical analysis of individual cells stimulated with TNF (adapted from Barken et al., 2005). Error bars are the mean ± SD. (D) Recordings from live individual cells transduced with overexpressing RelA and IκBα fusion proteins and stimulated with TNF, where each colored line represents the recording from one cell (adapted from Nelson et al., 2004). NF-κB nuclear localization predicted by a computational model (Hoffmann et al., 2002) with (E) and without (F) the IκBβ-mediated protective mechanism described in the text and in Phillips and Ghosh (1997).

shields it from IκBα-mediated nuclear export (Suyang et al., 1996; Phillips and Ghosh, 1997). This mechanism was included in our computational model that recapitulates NF-κB activation in response to TNF stimulation (Hoffmann et al., 2002), but our later studies were unable to observe the IκBβ effect in murine embryonic fibroblasts (MEFs), which are the cells for which the model was constructed (unpublished data). Removal of the mathematical term for this mechanism from the model resulted in highly oscillatory NF-κB responses (Fig. 1, E and F). Although genetic evidence points to important roles for IκBβ and -ε in regulating the dynamics of NF-κB signaling, the mechanisms by which they function remained unclear.

In this study, we investigated the dynamic behavior of all three canonical IκB isoforms, especially by contrasting IκBε and -β functions with that of IκBα. Our studies revealed that IκBε expression is, in fact, highly NF-κB inducible, and that it mediates functional negative feedback on NF-κB activity; however, it does so in antiphase to that of IκBα. Two antiphase negative feedbacks emerge as an important regulatory module that may be present for the dynamic control of signaling in other pathways as well.

Results and discussion

IκBε is TNF inducible via NF-κB

We constructed probes for RNase protection assays (RPAs) that allowed for the simultaneous quantitative monitoring of all three IκB mRNAs to characterize the regulation of IκBε and -β gene expression in response to stimulation. Analysis of the mRNA levels in MEFs that were stimulated with TNF confirmed that IκBα was strongly induced (Fig. 2 A). IκBβ showed only weak induction, which suggests that it is not a strong NF-κB–responsive gene in this cell type. Remarkably, IκBε transcription was highly induced by TNF stimulation, and quantitation of these results showed that IκBε was induced to a higher degree than IκBα (Fig. 2 B). Although the exact induction folds varied in replicate assays using separate MEF cell stocks (unpublished data), IκBε fold induction was consistently higher than that for IκBα. Furthermore, IκBε expression was also induced in response to LPS in MEFs, the macrophage cell line RAW264.7, and the B cell line 70Z (unpublished data), indicating that the dynamic control of its synthesis is not specific to TNF or to fibroblasts. Analysis of IκB mRNA levels in MEF cells deficient in NF-κB showed no induction of any of the three IκBs (Fig. 2 A).

The temporal profile of IκBα transcript induction during chronic TNF stimulation is well described (Scott et al., 1993). It shows rapid activation as early as 15 min, a peak within 1 h, and a slow attenuation over many hours. We observed a similar activation profile for IκBε induction, but were surprised to note a distinct 45-min onset delay (Fig. 2, A and B). This suggests that the IκBε promoter may involve a delay mechanism, such as a requirement for the activation of an NF-κB–responsive transcription factor (a feed-forward regulation). However, the inhibition of protein synthesis by cycloheximide did not attenuate transcriptional activation of either IκBα or -ε (Fig. 2 D and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200510155/DC1) and, thus, does not support the notion of feed-forward regulation.

It has been previously shown that transient TNF stimulation leads to transient nuclear NF-κB activity lasting only ∼60 min (Hoffmann et al., 2002). To determine whether short stimulation would efficiently induce IκBε expression, an RPA was performed on MEF cells that were stimulated for 15 min with TNF. The results show that IκBε transcription is still activated with an onset delay and with an induction profile similar to that of chronically stimulated cells (Fig. 2 C and Fig. S1). Because NF-κB activity is diminished when IκBε mRNA levels are still rising, and because cycloheximide treatment

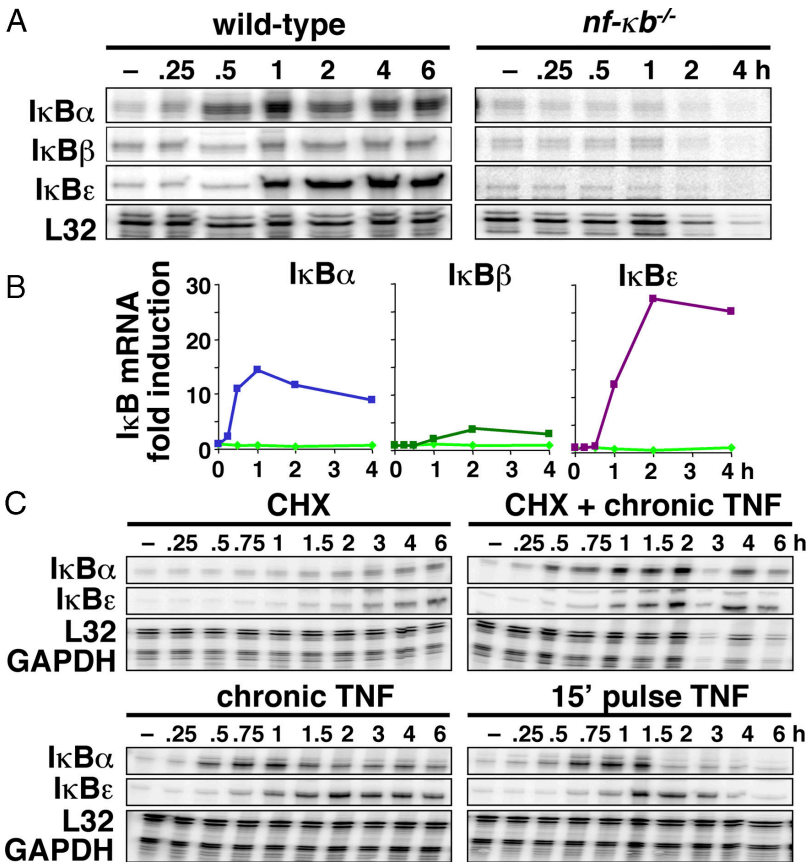

Figure 2. **IκB gene transcription in response to inflammatory stimulation.** (A) RPA revealing IκB mRNA levels in wild-type and NF-κB–deficient cells after chronic TNF stimulation. (B) IκB mRNA fold induction in wild-type (blue, dark green, and purple) and NF-κB–deficient (light green) stimulated cells that were normalized to L32 housekeeping gene expression. (C) IκB mRNA levels in wild-type cells in response to chronic treatment by cycloheximide and/or TNF and 15-min transient stimulation with TNF.

precludes a feed-forward mechanism, we suggest that the time delay in the activation of IκBε transcription occurs after NF-κB recruitment to the promoter. Further study is required to elucidate the mechanism of this delay.

Computational modeling reveals dynamic control mechanisms

NF-κB–responsive syntheses of IκBε and -β were added to the mathematical model to explore the functional consequences imparted by these negative feedback regulators upon NF-κB activity. To determine the kinetic parameter values for the inducible transcription of each isoform, the temporal responses of IκB transcription in the model were constructed such that the mRNA induction profiles calculated by the model correlated with our RPA data (Fig. 3 A). This required the fitting of parameters defining transcription and translation rates and mRNA stability. The new model also includes revised IκB protein degradation parameters from earlier studies (unpublished data).

The revised model recapitulates IκBα protein degradation immediately after IκB kinase (IKK) activation and rapid synthesis in response to NF-κB nuclear localization (Fig. 3, B and C). Chronic stimulation results in repeated IκBα protein degradation and synthesis (Fig. 3 B). In addition, the model shows delayed induction of IκBε and -β protein syntheses. The low inducibility of IκBβ transcription results in very low IκBβ protein synthesis, whereas the high inducibility of IκBε transcription results in notable accumulation of IκBε protein (Fig. 3 B).

Earlier studies revealed oscillatory NF-κB activity in cells lacking IκBβ and -ε (Hoffmann et al., 2002) and in cells in which NF-κB–inducible IκBα was overexpressed (Nelson et al., 2004), whereas in wild-type cells late NF-κB activity (beyond 2 h) was remarkably steady (Hoffmann et al., 2002). However, the underlying dampening mechanism that results in steadied late activity remained obscure. Because induced synthesis of IκBε is delayed, we reasoned that IκBε may mediate an antiphase negative feedback that provides effective dampening of IκBα-mediated oscillations. Indeed, our simulations of signaling modules lacking IκBε revealed oscillations in nuclear NF-κB that persist with a higher amplitude than those that contain IκBε and represent wild-type cells (Fig. 3 D). In contrast, simulations of cells lacking IκBβ do not show such aberrant oscillations, whereas systems lacking both IκBε and -β do.

We set out to examine these predictions experimentally, using nuclear extracts prepared from TNF-treated MEFs harboring genetic deficiencies for IκBε and/or -β. We measured NF-κB DNA-binding activity by electrophoretic mobility shift assay (EMSA; Fig. 3 E) and nuclear localization of the NF-κB protein RelA by Western blot (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200510155/DC1). In both assays, all four cell types exhibited fast induction of nuclear NF-κB in response to TNF stimulation by 15 and 30 min, a transient trough at 60–75 min, and subsequent recovery at 90–120 min. However, a second trough at 135–150 min was most pronounced in $i\kappa b\varepsilon^{-/-}$ and $i\kappa b\beta^{-/-}\varepsilon^{-/-}$ cells, as was a third trough at ~225 min. These studies suggest that negative feedback, provided by IκBε

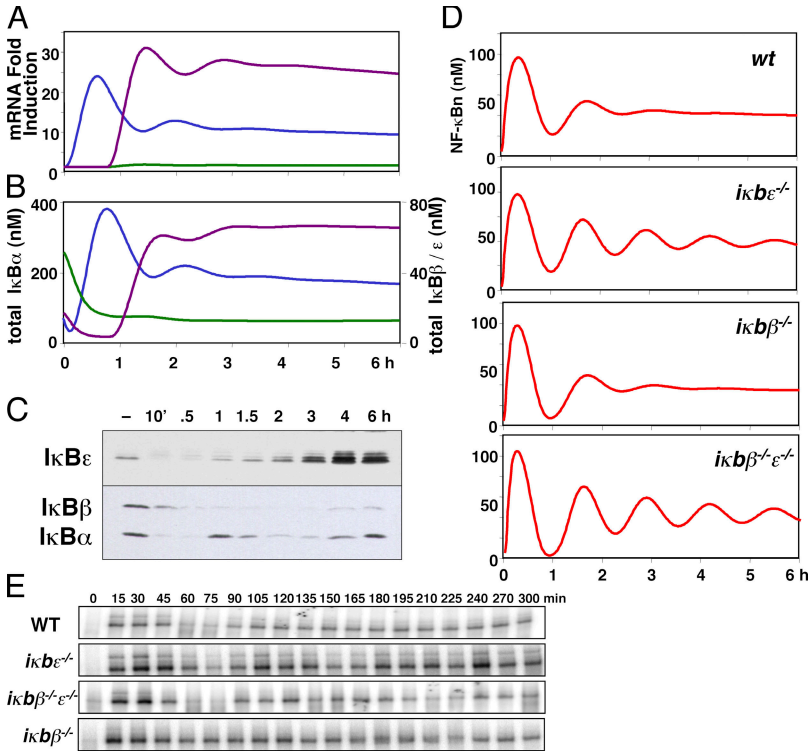

Figure 3. **Computational modeling of IκB mRNA and protein levels reveals a role for IκBε in regulating the dynamics of NF-κB activity in response stimulation.** The results of computational simulations of the fold induction of mRNA synthesis (A) and of the protein levels for IκBα (blue), IκBβ (green), and IκBε (purple) in wild-type cells (B) in response to persistent stimulation with TNF. (C) Western blots of IκB proteins in wild-type cells in response to persistent stimulation with TNF. The results of computational simulations (D) and EMSAs of nuclear NF-κB activity (E) in wild-type, $i\kappa b\varepsilon^{-/-}$, $i\kappa b\beta^{-/-}$, and $i\kappa b\beta^{-/-}\varepsilon^{-/-}$ cells in response to persistent TNF stimulation. Super-shift and oligonucleotide competition EMSAs are included in Fig. S2. Fig. S2 is available at http://www.jcb.org/cgi/content/full/jcb.200510155/DC1.

in antiphase to that of IκBα, is the primary mechanism that dampens the propensity for oscillations in NF-κB activity.

The regulatory motif consisting of two antiphase negative feedback systems may be present in other signaling pathways to control the dynamics of signal transduction, and variations in the relative strength of the two systems may provide for altered response dynamics to the same stimulus. A similar model has recently been proposed for two NF-κB–inducing signaling pathways emanating from the TLR4 receptor (Covert et al., 2005). In contrast to the two interacting negative feedback mechanisms, this model depicts the coupling of two positive oscillatory signals in an antiphase relationship that produces stable NF-κB activity in response to LPS stimulation in wild-type cells. As the temporal control of NF-κB activity determines NF-κB–responsive gene expression (Hoffmann et al., 2002; Nelson et al., 2004; Werner et al., 2005), the interaction of antiphase regulation by IκBα and -ε may contribute to the regulation of stimulus-specific and cell type–specific gene expression programs by modulating the dynamics of this transcription factor.

IκBε mediates postinduction repression of NF-κB activity and inflammatory gene expression

We used the computational model to identify conditions in which NF-κB–responsive IκBε expression would mediate negative feedback on stimulus-induced NF-κB activity and found that the most significant role for IκBε was in systems with reduced IκBα. To model such conditions, we used computational simulations to study the temporal profile of nuclear NF-κB in response to 15-min stimulation in systems lacking IκBα, -ε, or both (Fig. 4 A). Systems containing all three IκBs show rapid nuclear localization of NF-κB followed by removal from the nucleus within 1 h, as previously shown (Hoffmann et al., 2002). However, in systems lacking IκBα, we predicted effective down-regulation of NF-κB activity in the third hour and beyond. In this context, IκBε deficiency results in prolonged NF-κB activity, whereas in systems containing high IκBα expression it does not have an effect.

We used IκBα-deficient MEFs as a model for cell types that have reduced IκBα expression. These MEFs showed NF-κB activity to last ~3 h in response to 15-min transient TNF stimulation, after which it was dramatically attenuated (Fig. 4 B). In contrast, cells that were deficient in both IκBα and -ε showed a pronounced delay in attenuation, with NF-κB still present in the nucleus even at 6 h. Wild-type and IκBε-deficient cells are nearly indistinguishable, and both have strong NF-κB accumulation at 30 min and attenuation within 1 h. Collectively, these data strongly suggest that IκBε is responsible for the removal of NF-κB from the nucleus at late time points, allowing for dynamic functional interplay with the faster-acting feedback of IκBα.

Temporal control of NF-κB localization by IκBα was shown to control NF-κB–responsive gene expression not only quantitatively (Nelson et al., 2004) but also qualitatively (Hoffmann et al., 2002). To study the effects of IκBε-negative feedback on NF-κB–dependent gene expression, the transcription of five NF-κB–responsive genes was monitored by RPA after transient TNF stimulation in wild-type, $i\kappa b\alpha^{-/-}$, and $i\kappa b\alpha^{-/-}i\kappa b\varepsilon^{-/-}$ cells. The genes encoding TNF, G-CSF, and LIF are inducibly expressed in fibroblasts upon TNF stimulation, but mRNA levels return to baseline within 3 h in wild-type cells. In IκBα-deficient cells, these genes are attenuated within 4 h (Fig. 4 C). In this context, the loss of IκBε-negative feedback

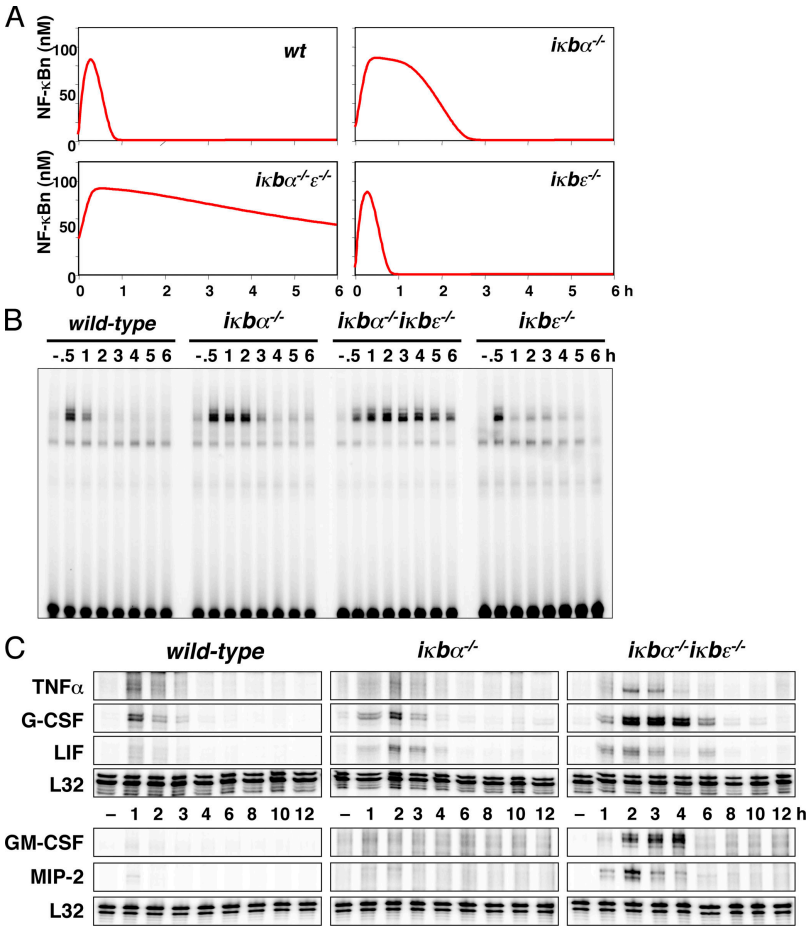

Figure 4. **IκBε mediates postinduction repression of NF-κB activity and inflammatory gene expression.** (A) Nuclear NF-κB activity as predicted by computational modeling after 15 min of TNF stimulation. (B) EMSA of nuclear NF-κB activity in extracts prepared at the indicated time points from wild-type, $i\kappa b\alpha^{-/-}$, $i\kappa b\alpha^{-/-}i\kappa b\varepsilon^{-/-}$, and $i\kappa b\varepsilon^{-/-}$ cells that were transiently stimulated for 15 min with 1 ng/ml TNF. (C) RPA reveals the mRNA levels over an extended time course of indicated NF-κB–responsive genes in wild-type, $i\kappa b\alpha^{-/-}$, and $i\kappa b\alpha^{-/-}i\kappa b\varepsilon^{-/-}$ cells that were transiently stimulated for 45 min with TNF.

results in a further delay in attenuation and quantitative deregulation of TNF, G-CSF, and LIF expression. Interestingly, the loss of both IκBα- and -ε-negative feedback has a dramatic qualitative effect for GM-CSF and MIP-2. Although these genes are not induced in wild-type or IκBα-deficient cells, both are strongly responsive to NF-κB activation when both IκBα- and -ε-negative feedbacks are absent. The data presented here demonstrate that IκBε-dependent negative feedback regulates the termination of NF-κB–responsive gene expression in both a quantitative (in the cases of TNF, G-CSF, and LIF) and qualitative (in the cases of GM-CSF and MIP-2) manner.

The functional interplay between the antiphase IκBα- and -ε-negative feedback responses may explain differences in NF-κB–dependent gene expression profiles seen in various cell types. In MEFs, IκBε-mediated negative feedback appears to be secondary to that provided by IκBα in response to transient inflammatory stimuli, and it is therefore assumed that IκBα controls the bulk of the NF-κB–responsive gene expression (Ghosh et al., 1998). However, the ratio of the abundance of IκBε in relation to IκBα is cell type–specific (Memet et al., 1999; Spiecker et al., 2000; Emmerich et al., 2003; Doerre et al., 2005), suggesting that IκBε may play a predominant role in NF-κB–responsive gene expression in particular cell types. Indeed, in vivo studies have shown that a deficiency of functional IκBε has physiological consequences (Memet et al., 1999; Spiecker et al., 2000; Emmerich et al., 2003; Doerre et al., 2005) and, thus, emphasize the notion that no IκB isoform functions on its own. To understand regulation of NF-κB activity in different cell types and in response to diverse stimuli, the interplay of all IκB isoforms within the IKK–IκB–NF-κB signaling module must be considered.

Our studies aimed to quantitatively characterize the temporal expression profiles of the three IκB isoforms and to examine their functional consequences on NF-κB regulation. Earlier studies showed inducible IκBε expression (Simeonidis et al., 1997; Whiteside et al., 1997). We have demonstrated that induction of IκBε is NF-κB dependent and functions to attenuate NF-κB activity and terminate NF-κB–responsive gene expression. Based on these three criteria we conclude that IκBε mediates bona fide functional negative feedback regulation on NF-κB activity. Importantly, our studies reveal that inducible expression of IκBε is delayed by 45 min with respect to that of IκBα, thus, creating a two–negative feedback regulatory module that critically controls the dynamics of NF-κB activity. We suggest that the relative strength of the two feedback mechanisms and their temporal relationship to each other may account for cell type–specific dynamic regulation of NF-κB activity.

Materials and methods

Cell culture

The immortalized MEF cells used were previously described (Hoffmann et al., 2002). Cells were grown to confluency in DME containing 10% bovine calf serum and starved for 24 h in media containing 0.5% bovine

calf serum. Stimulations were performed with 10 ng/ml TNF (Roche). Cells that were transiently stimulated with TNF were washed twice with 1× PBS after stimulation and returned to untreated media.

DNA-binding assays and Western blot

EMSAs were performed as previously described (Hoffmann et al., 2002). Western blots using whole-cell extracts were performed as previously described (Hoffmann et al., 2003). IκBα and -β antibodies were obtained from Santa Cruz Biotechnology, Inc. (SC-371 and SC-945, respectively). An antigen-purified polyclonal mouse antiserum raised against recombinant full-length mouse protein was used for IκBε.

RPA

Total cellular RNA was isolated from confluent and serum-starved cells with Trizol reagent (Invitrogen). Transcript levels were monitored with α-[^{32}P]UTP–labeled probes using a RiboQuant kit (BD Biosciences) according to the manufacturer's instructions. Data was obtained using a storage phosphor screen (GE Healthcare) and a variable mode imager (Typhoon 9400; GE Healthcare). Data was quantitated using ImageQuant software version 5.2 (GE Healthcare) by normalization to L32 and/or glyceraldehyde-3-phosphate dehydrogenase after local background subtraction. IκB probes were designed to select for mature mRNA species by spanning exon–exon junctions. The following primer pairs were used to amplify fragments from reverse-transcribed RNA: 5′-TCGCTCTTGTTGAAATGTGG-3′ and 5′TGGAGATTTTCCAGGGTCAG-3′ (IκBα); 5′-GCCCTTAGTCTTTGGCTACG-3′ and 5′-TCTCAGCCACCAACACTCCT-3′ (IκBβ); and 5′-GGCAGACAGCTTTCTCATCC-3′ and 5′-TGAGGTCGCAGTCTTCAATG-3′ (IκBε). G-CSF, LIF, MIP-2, TNF, L32, and glyceraldehyde-3-phosphate dehydrogenase probes were obtained from RiboQuant sets (BD Biosciences).

In silico studies

We previously constructed a computational model to describe NF-κB activation events in response to IKK activation by TNF (Hoffmann et al., 2002). This model comprises a singular NF-κB species, three IκB isoforms (IκBα, -β, and -ε), and IKK. Synthesis and degradation of the IκBs and cellular localization and interactions for all components were calculated using a system of ordinary differential equations. The model used in this study includes NF-κB–induced IκBε and -β transcription and was written in MatLab V7.0 (MathWorks) using previously described methods (Hoffmann et al., 2002). MatLab simulation files are available upon request.

Online supplemental material

Fig. S1 supports Fig. 2 C, with quantitation of IκBα and -ε gene induction profiles. Fig. S2 supports Fig. 3 E, with super-shift and oligonucleotide competition EMSAs, as well as nuclear westerns for RelA. Table S1 contains the computational model parameters and reactions.

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