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Authors
Fagerlund, R
Behar, M
Fortmann, KT
et al.

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Anatomy of a negative feedback loop: the case of IκBα

Riku Fagerlund1, Marcelo Behar1,†, Karen T. Fortmann1, Y. Eason Lin1,2,3, Jesse D. Vargas1,2,3 and Alexander Hoffmann1,2,3

The magnitude, duration and oscillation of cellular signalling pathway responses are often limited by negative feedback loops, defined as an ‘activator-induced inhibitor’ regulatory motif. Within the NFκB signalling pathway, a key negative feedback regulator is IκBα. We show here that, contrary to current understanding, NFκB-inducible expression is not sufficient for providing effective negative feedback. We then employ computational simulations of NFκB signalling to identify IκBα molecular properties that are critical for proper negative feedback control and test the resulting predictions in biochemical and single-cell live-imaging studies. We identified nuclear import and nuclear export of IκBα and the IκBα–NFκB complex, as well as the free IκBα half-life, as key determinants of post-induction repression of NFκB and the potential for subsequent reactivation. Our work emphasizes that negative feedback is an emergent systems property determined by multiple molecular and biophysical properties in addition to the required ‘activator-induced inhibitor’ relationship.

1. Introduction

Negative feedback control is a ubiquitous regulatory motif in many biological systems, critical to the maintenance of proper homeostasis, dynamic control in response to perturbations, or oscillatory patterns [1]. The defining feature of a negative feedback motif is an activator–inhibitor pair in which the activator induces expression or activity of the inhibitor. Indeed, many studies focus on the molecular mechanism(s) that provide(s) inducibility, often characterized by the fold change and any intrinsic delay. However, actual molecular circuits within cells are incompletely described by the activator-inducible inhibitor paradigm, as they may need to contend with physical realities within the cell such as the biochemistry of molecular interactions, sub-cellular compartmentalization or protein half-life. Thus, proper functioning of a negative feedback circuit may depend on biochemical properties other than the activator-responsive control of the inhibitor.

IκBα is a prominent negative feedback regulator in the NFκB signalling system [2,3]. IκBα directly controls the dynamics of the transcription factor NFκB, a central regulator of inflammatory and immune response gene expression [4,5]. Through its reversible sequestration of NFκB in the cytoplasm, IκBα not only controls the duration of NFκB activity [4,6] but also enables reactivation that can result in oscillatory dynamics observed both in population studies [4] and in single cells [7]. Mathematical models were shown to recapitulate these dynamic features [8,9], and reduced models have identified NFκB-responsive expression of IκBα as a key determinant of oscillatory dynamics [10–12]. The dynamics of NFκB signalling are stimulus-specific, and a critical determinant of inflammatory and immune gene expression programmes [13,14], prompting pioneering work to focus drug-targeting strategies on dynamical features to achieve superior specificity [15].

Here, we examine the molecular properties that confer IκBα’s ability to control NFκB dynamics. We find that while inducible expression of IκBα is required for proper NFκB dynamics [16], inducible expression is not sufficient as inducible expression of another IκB family member, IκBβ, is unable to...
support normal dynamical control of NFκB. This finding prompts us to characterize other IκBα properties that are required for proper negative feedback control of NFκB. Our study delineates how several molecular properties combine to produce the emergent systems property of dynamic negative feedback control of NFκB.
2. Results

2.1. NFKB-responsive transcriptional control is necessary but not sufficient for IκBα negative feedback

Studies of NFKB dynamic control by IκB family members have identified IκBα as the key negative feedback regulator due to its highly inducible NFKB-responsive promoter [2–4]. To characterize the role of NFKB-inducible expression, we complemented IκBα-deficient murine embryo fibroblasts (MEFs) with retroviral plasmids that express IκBα from either a constitutive (pBabe) or an NFKB-inducible (5xαB) promoter (figure 1a). Unlike pBabe-reconstituted cells, 5xαB IκBα reconstituted cells showed dynamic resyntheses profiles similar to endogenous IκBα in wild-type cells following stimulation with tumour necrosis factor (TNF) (figure 1b). Importantly, when we examined the control of NFKB activity by electrophoretic mobility shift assay (EMSA), we found that 5xαB cells showed post-induction repression and the transient trough of NFKB activity characteristic of wild-type cells, correcting the misregulation in IκBα-deficient cells, whereas cells constitutively expressing IκBα were unable to capture this response (figure 1b).

To test whether NFKB-inducible control was not only required but also sufficient for NFKB dynamic control, we complemented IκBα-deficient cells with a 5xαB retrovirus expressing IκBβ, a highly homologous IκB family member capable of inhibiting NFKB but not normally providing negative feedback. Interestingly, these cells did not show proper dynamic control of NFKB even though IκBβ expression was under NFKB control similar to IκBα (figure 1c; electronic supplementary material, figure S1A). However, when another known IκB negative feedback regulator, IκBe [17], was linked to this promoter, NFKB activity did show post-induction repression (electronic supplementary material, figure S1B). These results indicate that, despite the high degree of sequence homology, IκBα and IκBβ have distinct molecular properties that, along with differential gene expression control, render IκBα an effective negative feedback regulator but not IκBβ.

In order to confirm the validity of this conclusion, we obtained fibroblasts from a genetic knock-in mouse in which the IκBβ coding region was engineered to replace the IκBα open reading frame such that IκBβ expression was under the control of the endogenous IκBα promoter [18]. Remarkably, these so-called AKBI cells also failed to show proper NFKB post-induction attenuation despite highly inducible IκBβ expression (figure 1d; electronic supplementary material, figure S1c).

In order to examine translocation dynamics in single cells, and without the confounding contributions of other IκB family members, we generated IκBα/e−/−/IκBβ/e−/−/RelA/e−/−/3 T3 cells that lack all three classical NFKB inhibitors and RelA, and reconstituted them with a constitutively expressed fluorescent GFP-RelA and NFKB-inducible IκBα or IκBβ. Whereas reconstitution with IκBα resulted in transient NFKB activation in response to TNF treatment, defined by a trough at about 60 min, followed by a second phase in some cells (figure 1e), reconstitution with NFKB-inducible IκBβ resulted in sustained NFKB activation showing only slow and incomplete post-induction repression (figure 1f). Of note, in both conditions, the mean RelA nuclear localization profile of the collection of individual cells (figure 1c,d) closely resembled the population level in biochemical studies (figure 1c,d). These data clearly indicate that when expression of IκBα or IκBβ is driven by the same NFKB-responsive promoter, resulting in ostensibly similar expression profiles, only IκBα can provide effective dynamic negative feedback control on NFKB. Thus, inducible inhibitor expression in and of itself is not sufficient for proper negative feedback control of NFKB.

2.2. Mathematical modelling identifies multiple molecular properties of IκBα contributing to the negative feedback control of NFKB

IκBα has several characteristics—other than NFKB-dependent synthesis—that in principle may contribute to its negative feedback function, e.g., its nuclear import and export properties, as well as constitutive and signal-induced degradation of free and NFKB-bound IκBα (figure 2a). Here, we use a previously established in silico model of NFKB regulation to investigate the contributions of each of these processes to the control of dynamic NFKB signals. When normalized for maximum activity, we confirmed that partial inhibition of the NFKB-dependent synthesis of IκBα potently impaired post-induction attenuation, with 10% inhibition resulting in a 30% increase in the signalling level at 70 min (figure 2b, row 1, and 2c). However, we also found that partial inhibition of IκBα nuclear import had a similar effect with a 10% inhibition causing an 11% increase in signalling at 70 min (figure 2b, row 3). Weak inhibition of the degradation of free IκBα had little effect on post-attenuation induction, whereas stronger inhibition shifted
the peak of NFκB to later times resulting in a modest increase in late activity (90% inhibition caused 14% increase in signalling at 120 min, figure 2b, row 2). Partial inhibition of nuclear export or of signal-induced degradation of NFκB-bound IκBα also reduced the post-attenuation reactivation, with a 10% inhibition causing 1% and 9.5% decreased signalling at 120 min, respectively (figure 2b, rows 4 and 5).

To compare the contribution of these processes, we determined the sensitivity of NFκB activity at three specific times representing early, post-induction attenuation and late parts of the signal to various perturbations (figure 2c). We also quantified the global sensitivity to each perturbation as the root mean square deviation (RMSD) of the perturbed and unperturbed signals over 120 min, sampled at 1 min intervals (figure 2c(iv)). This analysis posits that IκBα-mediated post-induction attenuation of NFκB activity (figure 2c(ii)) is a function not only of the NFκB-dependent synthesis rate but also of IκBα’s nuclear import, as well as its constitutive degradation. It also predicts that nuclear export and IKK-dependent degradation of NFκB-bound IκBα are important for late post-attenuation signalling (figure 2c(iii)).

2.3. Experimental testing of model predictions: multiple IκBα properties contribute distinct characteristics to NFκB dynamic control

To test the computational predictions, we pursued a genetic perturbation approach. Previous work showed nuclear
import of IkBα to be mediated by an unconventional NLS [19,20]. Using this information, we reconstituted IkBα-deficient cells with an IkBα NLS mutant (IkBαNLSm: L110A,L115A,L117A,L120A). In DNA binding studies of IkBαNLSm cells, TNF induced NFkB activation comparable to that of wild-type IkBα cells (figure 3a), and although the resynthesis of IkBαNLSm protein was effectively induced by NFkB, the IkBαNLSm cells were defective for the rapid post-induction repression of NFkB. At 70 min, the signal in IkBαNLSm cells was 2.9-fold higher than in wild-type IkBα cells (considering the different basal levels). This is consistent with the threefold increase predicted by the model when the corresponding parameter is reduced to 35% of its wild-type value (figure 3a; electronic supplementary material, figure S2a). Consistent with population-level biochemical studies, IkBα−/− β−/− ε−/− RelA−/− cells expressing GFP-RelA showed that IkBαNLSm was defective in post-induction repression and cytoplasmic relocalization of NFkB in single cells (figure 3b). Despite the substantial heterogeneity in RelA cytoplasmic re-localization, the population mean closely resembles the population-level results obtained by EMSA. These data clearly show that the nuclear localization of IkBα is indispensable for proper termination of NFkB activity.

IkBα also relies on a nuclear export sequence (NES) for the efficient nuclear export of NFkB [21,22]. Mathematical modelling suggested strong inhibition of nuclear export would result in reduced late NFkB activity. To assess the role of IkBα nuclear export on the sub-cellular localization control of NFkB, we generated an NES mutant (IkBαNESm; L45A,L49A,J52A). Reconstituted cells expressing IkBαNESm were unable to produce the post-repression reactivation of NFkB characteristic of wild-type protein-controlled NFkB signalling (figure 3c; electronic supplementary material, figure S2b). The activity is qualitatively similar to the model prediction for a fivefold attenuation in the corresponding parameter. These results indicate that the nuclear export function of IkBα is crucial for the post-repression activation of NFkB signalling. Interestingly, the single-cell studies with the NES mutant revealed seemingly contradictory results (figure 3d), as most cells displayed sustained RelA nuclear localization. This apparent discrepancy is resolved by recognizing that: (1) the mutant localizes to the nucleus causing inhibition of NFkB activity but does not allow for NFkB export and reactivation, and (2) the biochemical assay detects DNA binding activity of free NFkB, whereas the single-cell imaging is a readout of NFkB localization only.

IkBα is known to have a very short half-life that is extended approximately twofold by an IkBαSM mutant (S283A, S288, T291A,S293A,T296A) [23,24]. When expressed in IkBα−/− cells from an NFkB-responsive promoter, IkBαSM achieved effective post-induction repression of NFkB activity (figure 3c; electronic supplementary material, figure S2c). However, the re-activation of NFkB was undetectable, consistent with computational predictions that indicated a signal close to basal level at 120 min when the corresponding parameter was reduced to 35% of its wild-type value. Similarly, in single live-cell studies, IkBαSM mediated efficient relocalization of RelA to the cytoplasm with a complete absence of late-phase activity (figure 3f).

3. Discussion

Given the well-documented role of NFkB activity in vital cellular processes, a number of mechanisms have evolved to ensure precise regulation of its activity. The IkBα negative feedback loop is a prominent NFkB regulatory mechanism, allowing for both post-induction repression and repeated or oscillatory bursts of activity, and is critical for providing complex dynamic control which is thought to mediate specificity in NFkB’s pleiotropic physiological functions. Prior studies have established that the NFkB-responsive IkBα promoter is critical for this negative feedback control [16], but it has remained unclear whether specific characteristics of the IkBα protein may be important as well. Indeed, biochemical studies presented here using MEFs derived from mice in which IkBβ was engineered into the IkBα locus (AKBI MEFs) clearly demonstrate that, even when IkBβ is under NFkB-transcriptional induction, it is unable to provide proper negative feedback. These data motivated our characterization of IkBα protein properties that contribute to proper negative feedback function. Our strategy was to complement IkBα−/− cells with retroviral transgenes providing for α- and β-responsive expression of engineered IkBα variants defective in specific molecular characteristics.

In addition to traditional biochemical approaches to study NFkB response and regulation, we examined NFkB response dynamics in single cells. Recent studies have characterized NFkB dynamics in single cells, but, to date, no studies have employed gene knock-out cells to probe underlying molecular mechanisms. Thus, regulatory control mechanisms identified at the biochemical/population level have yet to be reconciled with single-cell microscopy tracking studies that boast high temporal resolution and individual cellular histories. In this work, we employed a cell line lacking RelA and all canonical Ikβ proteins (IkBα−/− IkBβ−/− IkBe−/− RelA−/− cells), which we then reconstituted with NFkB-inducible Ikβ variants and fluorescent RelA reporter in order to examine the contributions of specific Ikβ protein characteristics.

Although the Ikβ proteins were first identified as cytoplasmic inhibitors, it has become clear that they play a major role in regulating nuclear NFkB. IkBα has been shown to be efficiently transported into the nucleus where it binds active NFkB dimers on the promoters of NFkB-activated genes and facilitates the dissociation of the transcription factor from DNA. Comparing mutants with wild-type Ikβ proteins, we were able to show the contributions of Ikβ inducible synthesis, nucleo-cytoplasmic transport and degradation control to the various aspects of the prototypical NFkB response; namely, duration and amplitude of initial NFkB activation, post-activation repression and post-repression re-activation of NFkB signalling. Our biochemical assays, together with single-cell studies, demonstrated that IkBα nuclear localization is indispensable for the rapid termination of NFkB activity. Specifically, we showed that an NES-deficient form of IkBα supported efficient induction and post-induction repression of NFkB DNA binding activity, but not the characteristic re-activation and second phase NFkB activity. The deficiency in NES function prevents the protein from efficiently returning NFkB to the cytoplasm for the next round of activation, maintaining an inactive IkBαNES-bound pool of NFkB in the nucleus. Finally, by employing an IkBα harbouring five mutations that confer stability, increasing the half-life of the normally rapidly turned over uncomplexed/free protein, we were able to show the importance of such rapid turnover in generating characteristic NFkB temporal profiles. In cells
Figure 3. Multiple IxBax properties contribute distinct characteristics to NFkB control. Nuclear localization of IxBax is required for the termination of NFkB activity (a,b). Nuclear export function of IxBax is required for post-repression activation of NFkB activity (c,d). IxBax protein half-life control is critical for sustained NFkB dynamics (e,f). IxBax<sup>+/−</sup> MEFS were reconstituted with NFkB-inducible wild-type or NLS mutant (a,b), NES mutant (c,d) or the 5M mutant (e,f) form of IxBax. The cells were treated with 1 ng ml<sup>−1</sup> of TNF and nuclear extracts were analysed by EMSA for NFkB activity and corresponding cytoplasmic extracts subjected to western blotting with indicated antibodies (a,c,e). Bar graphs show quantification of EMSA; curves are modelling the result of NFkB activity in single cells upon stimulation with 10 ng ml<sup>−1</sup> of TNF. Real-time fluorescent images of IxBax<sup>+/−</sup> β−/−<sub>b</sub>−/−<sub>e</sub>−/−<sub>RelA<sup>+/−</sup></sub> MEFS reconstituted with AcGFP1-RelA and NFkB-inducible IxBax NLS mutant (b), NLS mutant (d) or the 5M mutant (f) IxBax (showing cellular localization of RelA at indicated time points). Below the fluorescent images, single-cell traces show the ratio of nuclear to cytoplasmic localization of AcGFP1-RelA in fluorescent images (left) as well as the average curve and standard deviation of the single-cell traces (right).
expressing this IκBα5M form, we found a complete absence of second-phase activation, indicating that a low level of free IκBα protein (ensured by a short half-life) is required for this aspect of the response.

Our results demonstrate not only that IκBα feedback is dependent on NFκB-inducible synthesis but also that several other processes dependent on the molecular characteristics of the protein itself, for example import, export and half-life control, must be tuned in a coordinated manner to generate the hallmark features of NFκB signaling, namely post-induction repression and reactivation. By contrast, the IκBβ protein does not support proper negative feedback control even when expressed from IκBα’s promoter; we speculate that substantially reduced nuclear-cytoplasmic transport [25,26] may be a key underlying reason; a second characteristic that may play a role is IκBα’s but not IκBβ’s ability to strip NFκB off the DNA [27]. Indeed, these properties are not required for the NFκB dimer stabilization/chaperone function recently ascribed to IκBβ [28]. Our results illustrate the more general point: that negative feedback regulation in cells is a complex process that depends on multiple molecular properties beyond activator-induced expression [29,30]. These findings may well extend to other transcriptional networks as nuclear transport is a defining feature of many gene regulatory networks. Understanding the specific contributions of each process as well as their characteristic time scales is an important step for identifying effective druggable targets that may allow for correction of dynamic misregulation in cells associated with pathology [15].

4. Material and methods
4.1. Computational modelling
The response of the NFκB regulatory module was simulated using the computational ODE-based model described in [31]. In order to focus on regulatory mechanisms involving IκBα, the other IκB family members were removed. Following equilibration, TNF responses were simulated as in [31]. Time-course curves in figure 2 were generated by applying multipliers to the kinetic parameters corresponding to the reactions in figure 2r. The multiplier values were: 2.5, 2.5, 2.5, 2.5, 1, 0.05 (reactions 1, 2, 3 and 5) and 2, 2, 2, 2, 1 (reaction 4), reflecting different sensitivities for reaction 4. NFκB time courses are normalized to their peak value. Sensitivity ratios s(t) at a particular time t are defined as: (nucNFκBperturbed − nucNFκBunperturbed) / nucNFκBunperturbed, where nucNFκBperturbed/unperturbed are the normalized nuclear concentrations of NFκB at time t, obtained with a model with/without a multiplicative factor (0.9, 0.5, 0.1) for the indicated kinetic rate parameter (values shown in per cent units). The global average sensitivity in figure 2r was calculated as the RMS of the s(t) sampled at 1 min intervals between 1 and 120 min post-stimulation.

4.2. DNA constructs
NFκB-inducible IκBα and IκBβ constructs were generated in the self-inactivating (SIN) retrovirus backbone (HRSpuro) modified to express the IκBα or IκBβ transgene under the control of five tandem κB sites upstream of a minimal promoter. IκBα mutant forms were produced using site-directed mutagenesis. For live-cell studies, AcGFP1-RelA was fused to the N-terminus of RelA and the resulting construct was sub-cloned into the constitutively expressing retroviral plasmid pBabe-Hygro.

4.3. Cells and cell culture
Immortalized IκBα−/− MEFs were previously described [4] and IκBα−/− βB−/− e−/− RelA−/− MEFs were produced by interbreeding of the four individual mouse knock-out strains and harvesting E13.5 embryos, subjecting primary MEFs to the 3T3 protocol of repeated passage until a stably proliferating cell culture emerged. AKBI MEFs were a generous gift from BingBing Jiang (Boston University). MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 U penicillin/streptomycin (10378016; Life Technologies), 0.3 mg ml−1 glutamine and 10% fetal calf serum (complete medium). Plat-E cells [32] were maintained in complete medium containing blasticidin (10 μg ml−1) and puromycin (1 μg ml−1).

4.4. Retrovirus-mediated gene transduction
NFκB-inducible IκBα and AcGFP1-RelA constructs were transfected into Plat-E packaging cells pre-conditioned in antibiotic-free complete medium using poly(ethyleneamine). Supernatant was collected 48 h post-transfection, filtered and used to infect target cells with 4 μg ml−1 polybren to enhance infection efficiency (Sigma). Infected cells were selected with puromycin hydrochloride (Sigma) for IκBα and/or with hygromycin B (Invivogen) for the AcGFP1-RelA. Murine TNF (Roche) was used at 1 or 10 ng ml−1.

4.5. Biochemical analyses
Whole-cell extracts were prepared in radioimmunoprecipitation assay buffer with protease inhibitors and normalized for total protein before immunoblot analyses. Cytoplasmic and nuclear extracts for immunoblot analyses and EMSA, respectively, were prepared as previously described [4,24]. IκBα was probed with sc-371, IκBβ with sc-945 and α-tubulin with sc-5286. All antibodies were from Santa Cruz Biotechnology.

4.6. Microscopy
Cells were plated onto 35 mm glass bottom dishes (MatTek) or iBidi eight-well chambers (iBidi) 24 h prior to stimulation and immediate imaging. Images were acquired on an Axios Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 40×, 1.3 NA oil-immersion, or 20×, 0.8 NA air-immersion objective to a Coolsnap HQ2 CCD camera (Photometrics, Canada) using ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany). Environmental conditions were maintained in a humidified chamber at 37°C, 5% CO2 (Pecon, Germany). Quantitative image processing was performed using the FIJI distribution of IMAGE (NIH). All cells of each frame in the microscope image sampling experiments were measured for total fluorescence intensity. Time-course data were normalized by the minimum and maximum values to account for the varying overall intensities of different cells. The single-cell traces were averaged and error bars in the mean curves are the standard deviation from the mean.

Authors’ contributions. R.F. performed the experimental work, assisted by K.T.F., Y.E.L. and J.D.V. M.B. performed the computational modeling. R.F., J.D.V. and A.H. wrote the manuscript.

Competing interests. We have no competing interests.

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