NF- κ B dictates the degradation pathway of I κ B α

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I κ B proteins are known as the regulators of NF- κ B activity. They bind tightly to NF- κ B dimers, until stimulus-responsive N-terminal phosphorylation by IKK triggers their ubiquitination and proteasomal degradation. It is known that I κ B α is an unstable protein whose rapid degradation is slowed upon binding to NF- κ B, but it is not known what dynamic mechanisms control the steady-state level of total I κ B α . Here, we show clearly that two degradation pathways control the level of I κ B α . Free I κ B α degradation is not controlled by IKK or ubiquitination but intrinsically, by the C-terminal sequence known as the PEST domain. NF- κ B binding to I κ B α masks the PEST domain from proteasomal recognition, precluding ubiquitin-independent degradation; bound I κ B α then requires IKK phosphorylation and ubiquitination for slow basal degradation. We show the biological requirement for the fast degradation of the free I κ B α protein; alteration of free I κ B α degradation dampens NF- κ B activation. In addition, we find that both free and bound I κ B α are similar substrates for IKK, and the preferential phosphorylation of NF- κ B-bound I κ B α is due to stabilization of I κ B α by NF- κ B.

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Introduction

The NF- κ B family of dimeric transcription factors has an important function in many aspects of human physiology and disease. The family is composed of five members, RelA, RelB, p50, p52, and cRel that can form combinatorial hetero- and homodimers. The transcriptional activity of RelA- and cRel-containing dimers is tightly repressed by three inhibitors known as I κ B α , I κ B β and I κ B ϵ through the formation of stable I κ B–NF- κ B complexes. In response to extracellular stimuli, such as pro-inflammatory cytokines, the signal response domain of I κ B is phosphorylated by activated IKK (I κ B kinase), which leads to ubiquitination of I κ B by ubiquitin ligases and its degradation by the 26S proteasome

(Baldwin, 1996; Ghosh *et al*, 1998; Karin and Ben-Neriah, 2000), releasing NF- κ B to activate gene transcription.

The stimulus-induced degradation of I κ B α and activation of NF- κ B are well characterized; however, one important feature of NF- κ B regulation is its tight repression by I κ B in unstimulated cells. Elevated NF- κ B activity is associated with many human pathologies, including arthritis, atherosclerosis, and cancer (Courtois and Gilmore, 2006). Nevertheless, the manner by which NF- κ B activity is controlled in resting cells is unclear. It has been shown that I κ B proteins are continuously synthesized in uninduced cells; indeed, inhibition of protein synthesis activates NF- κ B and this activation requires basal IKK activity (Frankenberger *et al*, 1994; O'Dea *et al*, 2007). This implies that an IKK-dependent I κ B α degradation pathway exists in unstimulated cells and that continuous synthesis of I κ B α is essential to prevent basal NF- κ B activity. It is known that two distinct pools of I κ B α exist in cells; the larger I κ B pool is associated with NF- κ B (Scott *et al*, 1993) and the minor pool remains as a 'free' protein. Recent studies have revealed a three order of magnitude difference in the half-life of free and NF- κ B-bound I κ B α (O'Dea *et al*, 2007). Although free I κ B α is degraded rapidly, it has also been reported to be a poorer substrate for IKK than NF- κ B-bound I κ B α (Zandi *et al*, 1998). The apparent contradiction between inefficient IKK phosphorylation and the short half-life of free I κ B α remains unresolved.

Several studies have investigated the basal degradation pathways of both free and bound I κ B α , but have come to contradictory conclusions (Krappmann *et al*, 1996; Pando and Verma, 2000). One study showed that the basal degradation of both free and bound I κ B α occurs through the same pathway that do not require IKK phosphorylation or ubiquitination (Krappmann and Scheidereit, 1997). Later, others showed that basal degradation of the bound I κ B α did not require IKK phosphorylation, but could perhaps require ubiquitination. In addition, this study showed that free I κ B α required ubiquitination for degradation (Pando and Verma, 2000). Considering the potential significance of free I κ B α in NF- κ B regulation, we sought out to clearly understand the basal degradation mechanisms of the bound and free I κ B α to determine exactly how constitutive NF- κ B activity is regulated.

In the present study, we address these questions with new genetic tools and a mathematical model of the reactions that determine I κ B α metabolism and nuclear NF- κ B activity. We find that although free I κ B α can be a good substrate of IKK *in vivo*, rapid degradation of free I κ B α does not require IKK-mediated phosphorylation or lysine-targeted ubiquitination, and is instead regulated intrinsically by sequences in its C terminus. When the free I κ B α degradation pathway is altered, NF- κ B activation is severely dampened, proving the importance of a rapid free I κ B α degradation pathway. We address the functional significance of these differential degradation rates and pathways, and find that they are critical for allowing stimulus-responsive NF- κ B activation, while ensuring a low basal level of NF- κ B activity.

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Results

I κ B α is degraded independently of IKK phosphorylation and ubiquitination

Although I κ B α has been studied extensively as part of the I κ B–NF- κ B complex, the function and regulation of the free molecule remain unclear. It was reported previously that I κ B α is stabilized by NF- κ B (Rice and Ernst, 1993; Scott *et al*, 1993; O’Dea *et al*, 2007), but it has remained uncertain how I κ B α is degraded when it is not bound to NF- κ B. To characterize the degradation mechanism of I κ B α proteins, we used a retroviral transgenic system to introduce mutant forms of human I κ B α into mouse embryonic fibroblasts deficient in the NF- κ B proteins known to associate with it (Figure 1A). These *nfkb1*^{−/−}*rela*^{−/−}*crel*^{−/−} are referred to as *nfkb*^{−/−}. We find that when introduced into *nfkb*^{−/−} cells, wild-type (WT) human I κ B α , shows a remarkably short half-life of 10 min

or less, similar to the half-life of endogenous I κ B α in these *nfkb*^{−/−}-deficient cells (Figure 1B). It is important to note that the protein level of I κ B α in *nfkb*^{−/−} cells is much lower than I κ B α introduced into *ikba*^{−/−} cells, reconfirming the requirement for NF- κ B to stabilize the protein level of I κ B α (Supplementary Figure 1).

As I κ B α is known to be degraded in response to stimulation via IKK-mediated phosphorylation and specific ubiquitination, we first examined the role of these modifications by introducing specific mutations into I κ B α . Several reports have also looked into the basal degradation of the free pathway; however, there is still confusion as to the role of phosphorylation and ubiquitination in the basal degradation of free I κ B α (Krappmann *et al*, 1996; Pando and Verma, 2000). To eliminate IKK phosphorylation, we generated a transgenic *nfkb*^{−/−} cell line that expresses the S32A, S36A I κ B α mutant. The degradation rates were estimated by treating these cells

Figure 1 I κ B is an intrinsically unstable protein. (A) Schematic of I κ B α primary sequence. The signal response domain (SRD) is followed by the ankyrin repeat domain (ARD) and the PEST domain. IKK phosphorylation sites are indicated, as well as all lysines in I κ B α . (B) IKK phosphorylation mutants do not inhibit free I κ B α degradation. Left panel: western blot (WB) of WT and S32, 36A I κ B α from extracts of *nfkb*^{−/−} cells treated with cycloheximide (CHX) for the indicated times. Endogenous I κ B α and transgenic I κ B α are denoted by arrows. Right panel: this is presented graphically with three separate experiments plotted with error bars signifying $\pm$ s.e.m. (standard error of the mean). (○) Transgenic WT I κ B α and (◇) S32, 36A I κ B α . (C) Ubiquitination mutants do not slow free I κ B α degradation. Left panel: WB of WT I κ B α and different lysine mutants from cell extracts prepared as described in (B). Right panel: this is presented graphically with triplicate experiments plotted with error bars signifying $\pm$ s.e.m. (○) Transgenic WT I κ B α , (□) K21, 22R I κ B α and (△) KR9 I κ B α . (D) Free I κ B α is degraded by the proteasome. WB showing I κ B α in the extracts of transgenic cells were treated with MG132. All protein levels increase over time, which show that the proteasome is involved in the degradation of free I κ B α .

with cycloheximide, a translation inhibitor, and tracking the loss of I κ B α protein by western blot. We observed no difference in the apparent rates of degradation of the mutant and WT I κ B α (Figure 1B). To clearly understand whether ubiquitination is required for free I κ B α degradation, we created two different mutants: in one mutant, only the two lysines that have been shown to be ubiquitinated in stimulus-induced degradation of NF- κ B-bound I κ B α were mutated to arginines (K21, 22R). In the second mutant, all nine lysines present in the I κ B α protein were mutated to arginines (KR9) (Figure 1C). We observed that both K21R/K22R and KR9 degrade at a similar rate as WT I κ B α . In all, our results provide a clear answer that shows that neither IKK phosphorylation nor lysine ubiquitination is required for free I κ B α degradation.

As the proteasome is required to degrade ubiquitinated I κ B α in response to inflammatory stimuli, we wanted to test if the proteasome was also involved in the degradation of free, non-ubiquitinated I κ B α . When transgenic *nfkb*^{-/-} cells were treated with the proteasome inhibitor MG132, the amount of I κ B α increased rapidly in all cell lines (Figure 1D). This result has also been reproduced with a more specific proteasomal inhibitor, epoxomicin (Supplementary Figure 5). These results clearly show that even in the absence of IKK phosphorylation and ubiquitination the proteasome is essential for free I κ B α degradation. We conclude that I κ B α is an intrinsically unstable protein *in vivo*, which can be degraded in an ubiquitin-independent manner by the proteasome in unstimulated cells.

NF- κ B masks the intrinsic degradation signal of I κ B α

If I κ B α is intrinsically unstable *in vivo*, it may also be a good substrate for purified proteasome *in vitro*. We found that the purified 20S proteasome core, which degrades many unstable proteins such as p21 and ODC (Touitou *et al*, 2001; Chen *et al*, 2004; Alvarez-Castelao and Castano, 2005; Asher *et al*, 2005), also degrades I κ B α in a ubiquitin-independent manner (Figure 2A, lanes 1–4).

We next examined whether I κ B α bound to the NF- κ B dimer was also such a sensitive substrate for the 20S proteasome. We found that when I κ B α is complexed to recombinant RelA, the proteasome is no longer able to degrade it (Figure 2A, lanes 5–8). The intrinsic instability of I κ B α , which could be encoded in flexible regions of the protein, was apparently protected by NF- κ B. Both the N-terminal and the C-terminal PEST region of I κ B α are flexible and could potentially contribute to proteasomal recognition. Interestingly, our crystallographic and biochemical analysis of the I κ B α -NF- κ B complex suggests that the PEST region is protected from proteasomal degradation when bound to NF- κ B (Huxford *et al*, 1998; Phelps *et al*, 2000).

Does protection from ubiquitination-independent proteasomal degradation seen *in vitro* also occur *in vivo*? To that end, we introduced I κ B α transgenes into *ikba*^{-/-} NF- κ B-containing cells, and examined their degradation in the absence of stimulation. We found indeed that WT I κ B α transgenes introduced into *ikba*^{-/-} cells resulted in I κ B α proteins that had very long half-lives (~8 h versus ~10 min; compare Figure 1B versus top panel of Figure 2B). To test the effect of IKK phosphorylation and ubiquitination in the degradation of NF- κ B-bound I κ B α , *ikba*^{-/-} cells were reconstituted with mutants defective in IKK phosphorylation and ubiquitination. All of these mutants

are able to bind to NF- κ B in these cells, albeit with varying affinities, and responded as expected after treatment with TNF- α (Supplementary Figures 2 and 4). The levels of I κ B α observed represent bound I κ B α , since the half-life of bound I κ B α is much longer than the free form, and all of the free I κ B α is completely degraded within 60' of cycloheximide treatment. We observed that reconstituted I κ B α in *ikba*^{-/-} cells is only degraded when the IKK phosphorylation sites are intact (Figure 2B). When the preferred ubiquitination sites (K21, 22R) are mutated to arginine, degradation still occurs, which suggests that other lysines can be used for ubiquitination (Scherer *et al*, 1995). However, when all lysines are mutated (KR9), the degradation is slowed compared with WT I κ B α . These results suggest that in unstimulated cells, NF- κ B-bound I κ B α undergoes slow degradation that requires both IKK phosphorylation and ubiquitination. In addition, these observations point out that the same turnover pathway pertains to NF- κ B-bound I κ B α in unstimulated and stimulated cells. In contrast, free I κ B α turnover is determined intrinsically, independent of modifications such as phosphorylation or ubiquitination (Figure 2C). Again, we have clearly understood the modifications required for basal degradation of free and bound I κ B α .

The C-terminal PEST region regulates the degradation of free I κ B α

To examine which segment(s) of the I κ B α polypeptide determines the turnover rate *in vivo*, we generated three constructs that removed the flexible N and C termini to create I κ B α Δ N67, I κ B α Δ C303, and I κ B α Δ C288 (Figure 3A). *nfkb*^{-/-} cells stably expressing these mutants were generated and relative degradation rates were estimated using cycloheximide treatment. Removal of the N terminus did not alter the degradation rate (Figure 3B). The removal of the last 15 amino acids (I κ B α Δ C303) slowed the degradation rate (Figure 3C), although not to the extent of I κ B α Δ C288 (Figure 3D). The mRNA level of these truncated mutants were measured and are similar (Supplementary Figure 3). The segment (288–317) contains a PEST sequence, which encompasses residues 281–302. This observation is consistent with the PEST hypothesis, which states that the PEST sequence is responsible for protein turnover (Rogers *et al*, 1986). Within the PEST region, there are six serines and threonines that have been shown to be phosphorylated by casein kinase 2 (CK2) and several reports claim that this phosphorylation affects the turnover of I κ B α (Lin *et al*, 1996; McElhinny *et al*, 1996; Schwarz *et al*, 1996; Kato *et al*, 2003). We mutated these residues (S283, S288, T291, S293, T296 and T299) to alanine to generate the PESTA mutant. The PESTA mutant is degraded more slowly than WT I κ B α , but more rapidly than I κ B α Δ C288 (Figure 3D). These results suggest that phosphorylation of these residues contribute to free I κ B α degradation, but other residues within the PEST domain also contribute to the rapid degradation of free I κ B α . Overall, our experiments show an important role of the region encompassing residues 288–302 in the turnover of free I κ B α .

Efficient IKK phosphorylation of I κ B α does not require NF- κ B

One characteristic of the IKK-independent degradation pathway of free I κ B α could be that it is a poor substrate of IKK as

Figure 2 NF- κ B determines the degradation pathway of I κ B α . (A) NF- κ B protects I κ B α from proteasomal degradation *in vitro*. Top: purified 20S proteasome and I κ B α were incubated at 37°C, with or without purified p65. (I) represents the proteasome inhibitor, MG132. (B) I κ B α is highly stable *in vivo* in the presence of NF- κ B. Left panel: WB showing WT, IKK phosphorylation and ubiquitination-defective mutants introduced into *ikba*^{-/-}, where all NF- κ B subunits are present. Cells were treated with cycloheximide (CHX) for different lengths of time (up to 24 h) and the protein levels were visualized by WB. Right panel: this experiment was repeated twice and is represented graphically with error bars signifying $\pm$ s.e.m. ($\circ$) Transgenic WT I κ B α , ($\square$) K21, 22R I κ B α , ($\triangle$) KR9 I κ B α and ($\diamond$) S32, 36A I κ B α . (C) A model of NF- κ B repression by I κ B α in pre-stimulated cells. There are two processes that control I κ B α degradation. In the resting cell, basal IKK activity phosphorylates bound I κ B α and targets it for ubiquitin-dependent degradation. In addition, free I κ B α is continuously synthesized and degraded in an IKK- and Ub-independent mechanism. This keeps NF- κ B from being activated in the resting cell.

shown by *in vitro* experiments (Zandi *et al*, 1998). This substrate specificity could allow for accumulation of I κ B α without being phosphorylated and degraded. We therefore wondered if indeed free I κ B α is a poor substrate *in vivo*.

First, we carried out computational simulations with a mathematical model that recapitulates the kinetic reactions of IKK (Hoffmann *et al*, 2002; O'Dea *et al*, 2007). We increased the susceptibility of the free I κ B α protein for IKK-responsive degradation by increasing both IKK association

rate and catalytic rate constants to match that of NF- κ B-bound I κ B protein. Surprisingly, we found little effect on basal or TNF-induced NF- κ B activity (Figure 4A) (O'Dea *et al*, 2007). This simulation result suggests that low susceptibility to IKK-mediated phosphorylation and degradation of free I κ B proteins may not be functionally important for NF- κ B signalling.

We next explored in more detail whether a hypothetical mutant cell with an IKK that does not discriminate between

Figure 3 Rapid I κ B α turnover is conferred by intrinsic C-terminal sequences. (A) Schematic representation of various I κ B α constructs tested for their susceptibility to degradation as a free protein. (B) The N terminus of I κ B α does not determine degradation of I κ B α in the free form. Left panel: WB of I κ B α in extracts of cells expressing WT and Δ N67 I κ B α mutants in *nfkB*^{-/-} cells after treatment with cycloheximide (CHX). Right panel: triplicate experiments are represented graphically with error bars signifying $\pm$ s.e.m. Construction of stable cells and preparation of cell extracts were carried out as described in Figure 2. (●) Transgenic WT I κ B α and (▲) Δ N67 I κ B α . (C) The C terminus of I κ B α controls the rapid degradation of free I κ B α . Left panel: cells expressing transgenic I κ B α Δ C288 and I κ B α Δ C303 were treated with CHX for the indicated times and cell extracts were visualized by WB. Right panel: triplicate experiments representing WT I κ B α and I κ B α Δ C303 are represented graphically. (●) Transgenic WT I κ B α and (■) I κ B α Δ C303. (D) The PEST domain of I κ B α is responsible for high turnover rate of free I κ B α . Left panels (top and bottom): cells expressing transgenic WT I κ B α , I κ B α Δ C288 and PESTA I κ B α were treated with CHX for the indicated times and cell extracts were visualized by WB. (*) A nonspecific band, which can be used as a loading control. Right panel: triplicate experiments representing the relative degradation rates of WT I κ B α (black line), I κ B α Δ C288 (light grey line) and PESTA I κ B α (grey line) are represented graphically.

free and NF- κ B-bound I κ B proteins would have different signalling behaviour. Computational analysis did not reveal any significant differences in total I κ B protein levels in a simulated TNF time course (Figure 4B). Furthermore, the mutant cell showed very similar NF- κ B activation profiles in response to inflammatory stimuli in computational simulations (Figure 4C).

Results from these simulations prompted us to examine whether such substrate selectivity, reported for purified proteins *in vitro*, does in fact occur *in vivo*. Utilizing *nfkB*^{-/-} cells, we probed for the presence of phosphorylated I κ B protein in response to TNF. As these cells contain less total I κ B protein than their WT counterparts, we normalized the amount of protein loaded to the total amount of I κ B α present

in these cells. Surprisingly, we detected a strong band of P-I κ B protein in the *nfkB*^{-/-} cells, which we estimate to be about half of that detected in the transduced *ikba*^{-/-} cell extract diluted 10-fold to provide a roughly equal I κ B protein level (Figure 4D). This reduction in signal may be attributed to rapid (ubiquitin-independent) proteasomal turnover of free I κ B.

Our results suggest that free and bound I κ B may be similar substrates for phosphorylation by IKK and preference of IKK for a bound I κ B may be due to stabilization of I κ B by NF- κ B, rather than a difference in substrate recognition. If so, we reasoned that an I κ B protein that is intrinsically more stable may reveal IKK-induced degradation in the absence of NF- κ B. Indeed, computational simulations of I κ B protein

Figure 4 IKK phosphorylation of free I κ B α does not require NF- κ B, and has no functional consequence. **(A)** Computational simulations of basal NF- κ B activity or of peak NF- κ B activity after TNF stimulation. The IKK-dependent degradation rates of the free I κ B α s were increased to match the IKK-dependent degradation rates of the NF- κ B-bound I κ Bs. In the 'wild-type' (WT) model, the IKK-dependent turnover rate is 40-fold more efficient for NF- κ B-bound I κ B than for free I κ B α . This is decreased to 20-fold, and then finally to equal amounts of phosphorylation between free and bound I κ B α . **(B)** Computational simulations of nuclear NF- κ B levels in response to TNF for cells expressing WT IKK (black line) or a mutant IKK that does not discriminate between free I κ B α and bound I κ B α (grey line). **(C)** Computational simulations of I κ B α levels in response to TNF for cells expressing WT IKK (black line) or a mutant IKK that does not discriminate between free I κ B α and bound I κ B α (grey line). **(D)** Free I κ B α is phosphorylated in response to TNF- α . Top panel: WB of P-I κ B in response to TNF in *ikba* $^{-/-}$ and in *nfkb* $^{-/-}$ cells expressing WT I κ B α . The amount of protein in each time-course set was adjusted to equalize the amount of total I κ B α protein in the unstimulated cell extract. Bottom panel: the amount of phospho-I κ B α (normalized to total I κ B α levels) is compared between *nfkb* $^{-/-}$ and diluted extracts from *ikba* $^{-/-}$ cells after 10 min of stimulation. **(E)** Computational simulations of free I κ B α levels in response to TNF for *nfkb* $^{-/-}$ cells expressing either WT I κ B (black line) or a virtual I κ B α Δ C288 mutant with a five-fold decrease in the IKK-independent degradation rate of free I κ B α (grey line). **(F)** Free I κ B α Δ C288 is more responsive to TNF- α than WT I κ B α . WB of I κ B α Δ C288 and WT I κ B α after stimulation with 1 ng/ml TNF- α . (*) A nonspecific band, which can be used as a loading control.

levels in *nfkb* $^{-/-}$ cells indicated that although WT I κ B α levels are barely affected by TNF stimulation, the hyperstable I κ B α Δ C288 mutant is predicted to show a TNF-induced drop in protein level that may be discernible experimentally (Figure 4E). Indeed, western blotting of extracts made from TNF-induced *nfkb* $^{-/-}$ cells expressing I κ B α Δ C288 revealed that these protein levels decrease in response to TNF over time, whereas those of WT I κ B protein do not in these conditions (Figure 4F). These results show that NF- κ B binding to I κ B stabilizes the I κ B protein, and stabilization is what determines IKK-mediated degradation; however, the rate of IKK phosphorylation may be similar for free and NF- κ B-bound I κ B proteins. The functional specificity of IKK

instead is the result of the large differences in the rate of basal degradation between free and NF- κ B-bound I κ B α .

Rapid degradation of free I κ B α is critical for NF- κ B activation

If the amount of the free protein is altered significantly, this could have a detrimental effect on NF- κ B activation. To test this hypothesis, we introduced the longer half-life mutant I κ B α Δ C288 into *ikba* $^{-/-}$ cells and measured if a significant amount of this mutant remains free compared with WT I κ B α . RelA was immunoprecipitated and the amount of bound I κ B α was observed by western blot. Both I κ B α Δ C288 and WT I κ B α bound to RelA similarly (Figure 5A, lanes 3 and 4).

Figure 5 IKK-independent I κ B degradation is critical for signal responsiveness. **(A)** I κ B α Δ C288 accumulates as a free protein. Cells were incubated with an α -RelA antibody and immunoprecipitates and flow-through samples were analysed by WB. (*) Nonspecific bands. **(B)** NF- κ B activation is dampened due to stabilized free I κ B α . EMSA for NF- κ B activity in response to TNF stimulation in cells expressing WT I κ B α , I κ B α Δ C288, and PESTA I κ B α transgenes. **(C)** Quantitation of the EMSA in **(B)**. Two separate experiments are graphed. WT I κ B α is represented by black lines (solid and dashed), PESTA I κ B α is represented by grey lines (solid and dashed), and I κ B α Δ C288 is represented by light grey lines (solid and dashed). **(D)** Stimulus-induced IKK-dependent phosphorylation of I κ B α Δ C288 and PESTA I κ B α compared with WT I κ B α . Phosphorylation of I κ B α Δ C288 is slightly slower than WT I κ B α , whereas phosphorylation of PESTA I κ B α is slightly faster than WT I κ B α . Western blot showing phosphorylated I κ B α after stimulation with 1 ng/ml TNF- α . **(E)** Stabilized I κ B α degrades more slowly than WT I κ B α . Left panel: I κ B α Δ C288 and WT I κ B α transgenes in *ikba* $^{-/-}$ were treated with 1 ng/ml TNF- α and whole cell extracts were run on SDS-PAGE, and analysed by WB. Right panel: quantification of the left panel. WT I κ B α is represented by a black line, whereas I κ B α Δ C288 is represented by a light grey line.

The flow through was TCA precipitated and proteins were separated by SDS-PAGE followed by WB and probed for I κ B α and RelA. We clearly observe an excess of I κ B α Δ C288 compared with WT (Figure 5A, lanes 5 and 6), suggesting that we have indeed enhanced the free pool of I κ B α in the I κ B α Δ C288 mutant.

We then explored the functional effect of the I κ B α Δ C288 mutant with higher *in vivo* stability; measuring NF- κ B activation by electrophoretic mobility shift assay (EMSA) of nuclear extracts, we found that the I κ B α Δ C288 mutation caused a significant dampening of the NF- κ B activation profile. We also observed a lag in NF- κ B activation in cells expressing the I κ B α Δ C288 mutant. (Figure 5C, 1–30 min). To further understand how the stable I κ B α Δ C288 mutant negatively affects stimulus-dependent NF- κ B activation, we stimulated cells expressing WT I κ B α , I κ B α Δ C288, and I κ B α PESTA with TNF- α and probed for phosphorylated I κ B α . We find a slower phosphorylation rate of the I κ B α Δ C288 mutant compared with WT I κ B α . However, by 10' and 15', I κ B α Δ C288 is

eventually phosphorylated and degraded (Figures 5D and E). This slight shift in phosphorylation and degradation could account for lower NF- κ B activation at the early time point (20'). At the later time points, NF- κ B activation in cells expressing the I κ B α Δ C288 never reach the same maximum as WT I κ B α -expressing cells. This dampening effect is therefore due to the excess free I κ B α that never gets degraded (Figure 5E). Although the total amount of I κ B α Δ C288 phosphorylation is similar to WT I κ B α , the total amount of I κ B α Δ C288 mutant is significantly higher (Figure 5D). This explains why most, but not all of I κ B α Δ C288 is degraded even after 1 h of stimulation (Figure 5E). Our results thus suggest that the lag in NF- κ B activation might be due to the delay in IKK phosphorylation and that lower level of overall NF- κ B activity at all times is due to the excess amount of free non-degraded I κ B α . The PESTA I κ B α mutant (which has slower free I κ B α degradation) also has dampened NF- κ B activation (Figure 5B), and its phosphorylation is intact, if not more efficient than WT I κ B α (Figure 5E). Overall, our

results demonstrate for the first time that the rapid degradation pathway is essential for rapid and robust NF- κ B activation. Our combined computational and experimental results demonstrate that the constitutive degradation pathway of free I κ B α exists and is critical in allowing for appropriate activation of NF- κ B in response to a stimulus.

Discussion

Contrary to previous overexpression or cell-free biochemical-based analyses (Miyamoto *et al*, 1994; Krappmann *et al*, 1996; Lin *et al*, 1996; Schwarz *et al*, 1996; Van Antwerp and Verma, 1996; Pando and Verma, 2000; Tergaonkar *et al*, 2003; Alvarez-Castelao and Castano, 2005), the experimental work presented here using a clean genetic system delineates the free I κ B α degradation pathway as separate from the well-described IKK and β TrCP axis. We were able to (1) establish that I κ B α , which is not bound to NF- κ B, is an intrinsically unstable protein *in vivo*, (2) show that IKK phosphorylation and ubiquitination are not necessary for free I κ B α degradation, (3) identify the region of I κ B α responsible for the rapid degradation of free I κ B α , and (4) demonstrate that a stable free I κ B α negatively affects stimulus-dependent NF- κ B activation.

I κ B α degradation is rapid and regulated through the C-terminal PEST region

We show that the PEST sequence of free I κ B α is important for proteasomal degradation and that the primary degradation pathways of free and NF- κ B-bound I κ B α are different in unstimulated cells. Whereas bound I κ B α is degraded by the IKK-initiated ubiquitin-proteasome pathway in both stimulated and unstimulated cells, free I κ B α does not require phosphorylation by IKK nor ubiquitination for degradation. This conclusion is subject to the caveat that ubiquitination of the N terminus may be possible, but such a modification remains controversial (Sheaff *et al*, 2000; Bloom *et al*, 2003; Coulombe *et al*, 2004). Instead, we identified the PEST sequence containing C-terminal region of I κ B α as a determinant of its short half-life. In addition, we find (consistent with other reports) (Alvarez-Castelao and Castano, 2005) that the 20S core particle alone is able to degrade I κ B α *in vitro*, but we have been unable to establish its sufficiency *in vivo*. It is possible that other regulators of the 20S core particle are required for recognition of the PEST sequence, or perhaps even the entire 26S proteasome is responsible for the ubiquitin-independent degradation, as has been shown for p21 (Liu *et al*, 2006).

The current knowledge of Ub-independent protein degradation proposes that one of the criteria for Ub-independent protein degradation is the lack of high folding stability of the target substrate (Asher *et al*, 2006). I κ B α , not bound to NF- κ B, has a partially folded ARD and PEST sequence that is relatively unstructured (Croy *et al*, 2004). Upon binding to NF- κ B, both these regions of the protein become more folded (Huxford *et al*, 1998; Truhlar *et al*, 2006). In the X-ray crystal structure of the I κ B α -NF- κ B complex, the residues corresponding to most of the PEST region (residues 281–291) display clear electron density, and are involved in neutralizing the DNA-binding residues of the RelA/p50 heterodimer (Huxford *et al*, 1998). Chemical crosslinking experiments also revealed interactions between the PEST of I κ B α and the

DNA-binding domain of NF- κ B, which confirmed the structural studies (Phelps *et al*, 2000).

As such, we now understand the molecular interactions that show the interdependency between NF- κ B and I κ B α . Whereas I κ B proteins mask the DNA binding and nuclear localization sequences of NF- κ B, NF- κ B masks the intrinsic degradation signals in I κ B α preventing its rapid degradation.

The degradation kinetics of I κ B α determine IKK's functional specificity for NF- κ B-bound I κ B α

NF- κ B appears to direct the degradation of bound I κ B α through IKK-mediated N-terminal phosphorylation, as only NF- κ B-bound I κ B α levels drop significantly in response to IKK-inducing stimuli. Yet our analysis of I κ B α phosphorylation in NF- κ B-deficient cells indicates that free I κ B α is also a good substrate for IKK. We find that I κ B α susceptibility to IKK-mediated degradation is dependent on its stabilization by NF- κ B. Indeed, stabilizing I κ B by introducing appropriate mutations in its C terminus also sensitizes the protein for IKK-mediated degradation. Our results refine a previous model that suggests that negative feedback regulation by I κ B α requires a build-up of the free protein that is not sensitive to IKK-mediated degradation (Zandi *et al*, 1998). Since then, TNF-induced IKK activity was shown to be attenuated at 25 min after stimulation, which may allow for the build-up of newly synthesized I κ B α (Werner *et al*, 2005; Cheong *et al*, 2006).

Instead, the functional specificity of IKK for NF- κ B-bound I κ B is achieved through a large difference in basal degradation rates rather than a preference of the kinase for the NF- κ B-bound protein. As of yet, there is no information on the interaction between NF- κ B and IKK nor any data suggesting that there are conformational changes of the I κ B N terminus triggered by NF- κ B binding, either of which would provide a platform for IKK preferential phosphorylation. Thus, NF- κ B determines the fate of I κ B α through stabilization; not only does the physical interaction with NF- κ B preclude its rapid degradation, this stabilization also allows phosphorylation by IKK and thereby stimulus-responsive NF- κ B activation.

The instability of I κ B tunes the cellular responsiveness to inflammatory stimuli

Although stabilization of I κ B by NF- κ B is a hallmark of the NF- κ B signalling module, we found that stabilization of free I κ B α through disruption of the free I κ B α degradation pathway can inhibit NF- κ B activation. Although free I κ B α is not responsive to stimulus, this degradation pathway is nonetheless a determinant of stimulus-responsive NF- κ B signalling (Figure 6). This finding may be rationalized by the fact that high constitutive I κ B transcription and translation ensure an excess of I κ B synthesis. High degradation rates of free I κ B α ensure a low level of excess I κ B α , which is estimated to be about 15% of the total (Rice and Ernst, 1993; O'Dea *et al*, 2007). Tuning the level of free I κ B α in the cell by controlling either synthesis or degradation may therefore provide opportunities for signalling cross-talk. That is, non-inflammatory signals, such as those derived from environmental or metabolic stress conditions, may affect the responsiveness of the cell to inflammatory stimuli that regulate NF- κ B via IKK by affecting either the

Figure 6 A final model of the degradation pathways controlling I κ B α in basal and stimulated cells. In the resting cell, enough I κ B α is synthesized that it can rebind any NF- κ B released due to slow basal IKK activity. Free I κ B α is degraded very rapidly, and only represents ~15% of the total I κ B α in the cell (transparent I κ B α). When I κ B α binds to NF- κ B, it is stabilized, and must go through IKK-dependent phosphorylation and degradation (bold I κ B α). Upon stimulation, the activity of IKK is increased such that most (if not all) I κ B α is rapidly degraded and allows for NF- κ B activation. Free I κ B α must be continuously degraded to allow for this rapid and robust NF- κ B activation.

free or bound degradation pathway. Finally, as demonstrated in our study, using a combined approach in which quantitative biochemical studies interface with mathematical modelling may therefore help understand the differential responsiveness of cells in stressed conditions often found in pathological contexts.

Materials and methods

Cell culture

Immortalized 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 100 U/ml penicillin-streptomycin-glutamine. Cells were stimulated with various amounts of TNF- α (Roche Biochemicals). Cycloheximide was used at 10 μ g/ml resuspended in 50% EtOH (EMD Biosciences). For proteasome inhibition, 50 μ M MG132 and 10 μ M epoxomicin were used for various times (Calbiochem). 293T cells were grown in DMEM supplemented with 10% fetal bovine serum.

DNA constructs

I κ B α constructs were cloned into the retrovirus vector pBabe-puro between the restriction sites *Eco*RI and *Sall*. Mutagenesis reactions were performed with the Stratagene Quickchange Mutagenesis kit.

Production of virus

293T cells were transiently transfected with either CaPO₄ or Lipofectamine 2000. Retroviral vector (8 μ g) was co-transfected with pCl-Eco (Imgenex) and cells were allowed to grow for 40–48 h. The supernatant was then filtered and placed onto the target 3T3 cells along with 8 μ g/ml polybrene (Sigma). These cells then grew for another 48 h before selection with 10 μ g/ml puromycin (Calbiochem).

Cell stimulation and western blot analysis

After treatment with cycloheximide or TNF- α , cells were lysed in a modified RIPA buffer. Approximately 40 μ g of each cell extract was separated on a 12.5% SDS-PAGE and transferred to a nitrocellulose

membrane. I κ B α was probed with either sc-371 (Santa Cruz Biotechnologies) or sc-203 followed by anti-rabbit HRP conjugate. Quantification of western blots was performed with ImageQuant TL (Amersham Biosciences).

Immunoprecipitation and TCA precipitation

Approximately 1 mg of total cellular protein in modified RIPA buffer was precleared with 40 μ g of protein G agarose beads, and incubated overnight with α -RelA (sc-372-G) at 4°C. Immunoprecipitates were captured with protein G beads, washed three times with 100 mM Tris pH 7.5, 250 mM NaCl and 1% Triton-X, boiled and run on a 12.5% SDS-PAGE gel. After immunoprecipitation, the flow through was precipitated with 5% TCA, and spun for 10' at 40°C. The supernatant was discarded, and the pellet was washed 3 $\times$ with 1 ml of acetone. The pellet was then dried and resuspended in 4 $\times$ SDS dye, and run on a 12.5% SDS-PAGE gel.

Proteasome degradation assay

Purified I κ B α (residues 6–317) and p65 (residues 1–325) were purified as previously described (Huxford *et al*, 1998). The 20S proteasome was a gift from Dr Rechsteiner and Dr Pratt, University of Utah. For the degradation reactions, the proteins and the proteasome were mixed in a molar ratio of 25:1 in a buffer containing 20 mM Tris pH 7.0, 250 mM NaCl, 10 mM MgCl₂ and 1 mM DTT at 37°C. Samples were removed at various time points and the reaction was stopped by adding SDS-PAGE loading dye and boiling. Protein bands were separated by SDS-PAGE and visualized by western blotting. 1 mM of MG132 was used for inhibition of the proteasome (Calbiochem).

EMSA

Following stimulation with TNF- α , cells were washed twice with ice-cold phosphate-buffered saline + 1 mM EDTA and collected. The pellet was resuspended in 100 μ l CE buffer (10 mM Hepes-KOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 0.54% NP-40, 1 mM DTT and 1 mM PMSF) and vortexed for lysis. Nuclei were pelleted at 4000g, resuspended in 30 μ l NE buffer (250 mM Tris (pH 7.8), 60 mM KCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) and lysed by three freeze-thaw cycles. Nuclear lysates were cleared by 14 000g centrifugation and protein concentrations were normalized through Bradford

assay. Total nuclear protein (2.5 μ l) was reacted at room temperature for 15 min with 0.01 pmol of 32P-labeled 38-bp double-stranded oligonucleotide containing two consensus κ B sites: (GCTACAAGG GACTTTCGCTGGGGACTTTCAGGAGG) in binding buffer (10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA and 0.1 μ g/ μ l polydIdC), for a total reaction volume of 6 μ l. Complexes were resolved on a non-denaturing 5% acrylamide (30:0.8) gel containing 5% glycerol and 1 $\times$ TGE (24.8 mM Tris, 190 mM glycine and 1 mM EDTA), and were visualized using a PhosphorImager (Molecular Dynamics).

RNase protection assay

Total cellular RNA was isolated from confluent with Trizol reagent (Invitrogen). Transcript levels were monitored with α -[³²P]UTP probes using a RiboQuant kit (BD Biosciences) according to the manufacturer's instructions. Data were obtained using a storage phosphor screen (GE Healthcare) and a variable mode imager (Typhoon 9400; GE Healthcare). Data were quantitated using ImageQuant TL (Amersham Biosciences) by normalization to L32 and/or glyceraldehyde-3-phosphate dehydrogenase after local background subtraction. I κ B probes were designed to select for mature mRNA species by spanning exon-exon junctions. L32, and glyceraldehyde-3-phosphate dehydrogenase probes were obtained from RiboQuant sets (BD Biosciences).

In vitro IKK kinase assay

After TNF stimulation, cytoplasmic extracts were isolated from cells as described above using 200 μ l IKK CE buffer (10 mM Hepes-KOH (pH 7.9), 250 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.2% Tween 20, 2 mM DTT, 1 mM PMSF, 20 mM β -glycerophosphate, 10 mM NaF and 0.1 mM Na₃VO₄), and were normalized through Bradford assay. Cytoplasmic extracts (100 μ l) were incubated with 1 μ g IKK γ monoclonal antibody (BD Pharmingen) for 2 h at 4°C, and then with protein G agarose-conjugated beads (Amersham Biosciences) for 1 h at 4°C. After washing with IKK CE buffer twice and kinase buffer (20 mM Hepes (pH 7.7), 100 mM NaCl, 10 mM MgCl₂, 2 mM DTT, 1 mM PMSF, 20 mM β -glycerophosphate, 10 mM NaF and 0.1 mM Na₃VO₄) once, the beads were incubated with 20 μ l kinase buffer containing 20 μ M

adenosine 5'-triphosphate (ATP), 10 μ Ci [³²P]ATP and 0.5 μ g bacterially expressed GST-I κ B α (1-54) substrate at 30°C for 30 min. The reaction was resolved by 10% SDS-PAGE and was visualized and quantified by PhosphorImager (Molecular Dynamics). To normalize kinase activities, a portion of the SDS gel (175-50 kDa) was transferred to PVDF (Amersham Biosciences) and probed for IKK α (Santa Cruz Biotechnologies) (Werner *et al*, 2005).

Computational modelling

A modified version (version 2.1) of a mathematical model of the IKK-I κ B-NF- κ B signalling module (Barken D, unpublished results) based on the previously described version 2.0 (Werner *et al*, 2005), was used for all simulations. Simulations were carried out in Matlab version 2007a (Mathworks) using the built-in ode15s solver at default settings. To simulate *ib*^{-/-} cells expressing exogenous I κ B α , simulations were run with the NF- κ B-inducible I κ B synthesis set to zero. To simulate *nfb*^{-/-} cells, simulations were run with NF- κ B values set to zero. When altering the IKK-dependent degradation rates, both the association rate of I κ B with IKK and the degradation rate of I κ B by IKK were altered.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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