UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Molecular and Functional Characterization of a Degron in IkappaBalpha

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Chemistry

by

Karen Teresa Fortmann

Committee in charge:

Professor Gourisankar Ghosh, Co-Chair
Professor Alexander Hoffmann, Co-Chair
Professor Daniel Donoghue
Professor Randolph Hampton
Professor Elizabeth Komives
Professor Katja Lindenberg

2014

The Dissertation of Karen Teresa Fortmann is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Co-Chair

University of California, San Diego

2014

DEDICATION

To my family, especially my parents thank you for giving me life and asking for nothing in return. I may not be Dr. Schuerenberg, but I'll always be your little girl.

To my husband, who makes me smile and laugh constantly, I can't wait to go on many more adventures with you. Thank you for you patience and unconditional love.

To Dr. Huxford, my first biochemistry professor at SDSU, your excitement for science and teaching is the reason I went to graduate school.

EPIGRAPH

"Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning."

Albert Einstein

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LIST OF ABBREVIATIONS

ARD	Ankyrin Repeat Domain
AR	Ankyrin Repeat
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
Bcl-3	B-cell lymphoma 3-encoded protein
CHX	Cycloheximide
DMEM	Dulbecco modified essential media
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E3	Ubiquitin ligase
EDTA	Ethylenediamenetetracetic acid
EMSA	Electrophoretic mobility shift assay
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia inducible factor alpha
IκB	Inhibitor of NFκB
IκBα	I kappaB alpha
IκBβ	I kappaB beta
IκBδ	I kappaB delta
IκBε	I kappaB epsilon
IκBζ	I kappaB zeta
IKK	Inhibitor of kappB kinase
IKKα	Inhibitor of kappaB kinase alpha
IKKβ	Inhibitor of kappaB kinase beta
IKKγ	Inhibitor of kappaB kinase gamma
IKKε	Inhibitor of kappaB kinase epsilon
IFNγ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor

kDa	KiloDalton
KOH	Potassium hydroxide
LPS	Lipopolysaccharide
MEF	Murine Embryonic Fibroblast
MG-132	Z-Leu-Leu-Leu-aldehyde
NaCl	Sodium Chloride
NaDOC	Sodium Deoxycholate
NEMO	NFkappaB Essential Modulator
NFκB	Nuclear Factor kappaB
NLS	Nuclear localization sequence
NMR	Nuclear magnetic resonance
NP-40	Nonyl phenoxypolyethoxylethanol
ODC	Ornithine decarboxylase
PA28	Proteasome activator 28
PAGE	Polyacrylamide gel electrophoresis
PEI	Polyethylenimine
PEST	Proline, glutamic acid, serine, threonine rich region
PMSF	Phenylmethylsulfonyl fluoride
RA	Rheumatoid arthritis
RHD	Rel Homology Domain
SDS	Sodium dodecyl sulfate
SRD	Signal responsive domain
TAD	Trancriptional activation domain
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TNF-R	Tumor necrosis factor receptor
TRAF	TNFR-associated factor
Tris	tris(hydroxymethyl)aminomethane
UPR	Unfolded protein response
WT	Wild-type

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ACKNOWLEDGEMENTS

This dissertation would not be possible without the help and advice of a large number of people. I would fist like to thank my advisor and mentor, Professor Alexander Hoffmann. Alex has had a profound impact on my scientific career. He has provided a wonderful environment to do research in and has provided the lab with a generous amount of funding that has granted us the luxury of focusing primarily on our research. He has taught me how to think more objectively and his enthusiasm for his work has helped shape me as a scientist. I would like to think that I also had a significant impact on Alex; I do not think he had ever thought he would have such a stubborn and emotional trainee to guide and I hope that I never caused him too much trouble.

I would like to thank my committee members – Drs. Gourisankar Ghosh, Daniel Donoghue, Randolph Hampton, Elizabeth Komives, and Katja Lindenberg, for taking the time and interest in my education. I would especially like to thank Gouri Ghosh, my Co-Chair, and Elizabeth Komives, who are both members and collaborators for the Program Project Grant that has funded my research for the last 5 years. Their support and guidance has been instrumental throughout my graduate work. Both Professors have been wonderful to talk to not only about the science that we love so much, but also about life and career decisions. I genuinely appreciate all the help and advice that my committee has provided.

I would also like to acknowledge the past and future members of the Signaling Systems Labaoratory (SSL). I could not have asked for a more nurturing and

pleasurable lab to work in. We spend a lot of time in lab, and coming into a work place that you're excited to be a part of is an amazing feeling. There have been quite a few people pass through the doors of this lab but I would especially like to thank the following lab members. Ellen O'Dea Mercado was a graduate student who had the misfortune of training me when I joined the lab. It was her work on free IκBα degradation and more importantly her passion for science that that initiated my graduate research project. She was a brilliant scientist who I still aspire to be like. When I first started in the lab, I looked up to Bärbel Schröfelbauer as a role model for her tireless work ethic and the ease at which she had with science. She was a phenomenal scientist and teacher and taught me numerous tricks of the trade. Diana Rios, for having the patience to show me how to run an EMSA multiple times before I got the hang of it. Rachel Tsui, for being the same year as me in the program and repeating the mantra "we will graduate and find jobs". More personally I want to thank Diana and Rachel for being such good friends and listening to rants when things inside (or outside) of lab got frustrating or went wrong. Kim Ngo was the lab manager when I first joined the lab and later joined as a graduate student, she was one my office mates and provided me with snacks and an outlet for frustration as well as showing me tricks and tools along the way. I especially want to thank my undergraduate mentee, Russell Lewis, who not only helped motivate me by seeing how diligent he was in lab, but also contributed to my graduate work and this dissertation. Lastly, I want to acknowledge Paul Loriaux and Maxim Shokhirev for always being supportive of stress relief by way of the pub and every other Hoffmann lab member; you have all had a significant impact on my life.

The last group of people that deserve credit is my family and friends. I want to thank them for their unwavering support and understanding. My parents who have given me life and asked for nothing in return, their pride and excitement in my Ph.D. reminds me to recognize my accomplishments and take a step back to remember this special time in my life. My friends for prompting me to consider activities outside of the laboratory, I cannot imagine my life with out the stress relief that my friends bring. My husband, Brian, for being with me every crazy step of the way and giving me a solid support system in life and in school. Graduate school is a rather selfish endeavor and Brian has never questioned my decision to get a Ph.D. He has shown me how to look at life more optimistically and has talked me out of quitting more times than I would care to admit. Moreover, he has reminded me that we are making our journey through life together and that nothing, not even a bad day in the lab, can stop us.

I have had the pleasure of collaborating with a couple of people who have contributed directly to the work presented in this dissertation. The work presented in Chapter 2 and Chapter 3 has culminated in a manuscript, where I performed a majority of the experimental work and Russell Lewis provided experimental data, as well as, cloning, and Jesse Vargas provided microscopy training; Alexander Hoffmann will be the corresponding author. The work in Chapters 4 contains preliminary data and is in preparation to be submitted for publication. Specific contributions will be acknowledged at the close of each chapter.

VITA

2009	Bachelor of Science, San Diego State University
2011	Master of Science, University of California, San Diego
2014	Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

Fortmann, K.T, Lewis, R.D., and Hoffmann, A. (2014) A regulated, ubiquitin-independent degron in IκBα. *In Preparation*

Mercado, E., Mitchell, S., **Fortmann, K.T.,** Hoffmann, A. (2014) Type I and type II interferons potentiate NFκB activation via distinct mechanisms. *In preparation*

Fagerlund, R., Behar, M., **Fortmann, K.T.,** Vargas, J.D., Hoffmann, A. (2014) Anatomy of a negative feedback loop: the case of IκBα. *In preparation*

Wojciak, J.M., Zhu, N., **Schuerenberg, K.T**., Moreno, K., Shestowksy, W.S., Hiraiwa, M., Hansen, G., Sabbadini, R., and Huxford T. (2009).
The crystal structure of sphingosine-1-phosphate in complex with a Fab fragment reveals metal ion bridging of a humanized monoclonal antibody and its antigen. *Proceedings of the National Academy of Sciences*

ABSTRACT OF THE DISSERTATION

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by

Karen Teresa Fortmann

Doctor of Philosophy in Chemistry

University of California, San Diego, 2014

Professor Gourisankar Ghosh, Co-Chair

Professor Alexander Hoffmann, Co-Chair

Cells are capable of rapidly responding to changes in their environment, including the presence of pathogens or noxious conditions. Molecular signaling pathways that regulate these responses show highly dynamic patterns of activity. Indeed, the controls of half-life and protein degradation are hallmarks of many signaling pathways. In the case of the NFκB regulatory pathway, the key mediator of inflammatory responses, the inhibitor proteins IκBα, -β, and -ε are known to be regulated by signal-responsive mechanisms freeing NFκB. Recent work also indicates that IκBα is synthesized in excess in resting cells to ensure that NFκB activity remains

effectively inhibited. Rapid degradation of the excess IκB is critical so that NFκB activation can proceed rapidly when inflammatory responses are needed.

Cellular protein degradation is catalyzed by the proteasome, a large molecular machine. Proteins are usually targeted for degradation by a specific post-translational modification, the covalent attachment of the small molecule ubiquitin. In this dissertation I report that IκBα does not require such modifications for its rapid degradation, but instead relies on a specific amino-acid sequence, termed a degron, that targets even heterologous proteins to the proteasome. Interestingly these amino acids are buried within the IκBα-NFκB complex, thus stabilizing IκBα, and allowing it to effectively inhibit NFκB. This work describes a novel determinant of protein half-life control and shows that it is amenable to regulation by a post-translational mechanism to stabilize the target protein.

In examining the other IκB family members, I show that while IκBβ may undergo similar control, IκBε is in fact a stable protein that maybe less suited to ensure NFκB inhibition in resting cells.

Chapter 1:

Introduction

THE DYNAMICS OF SIGNALING

Protein synthesis is not only required when cells are growing or reproducing: protein turnover, the process by which proteins are synthesized and degraded, is a constitutive cellular process. As protein turnover is critical for cellular homeostasis and response to environmental changes, determining how and when a given protein should be degraded is a fundamental decision for a cell. Different proteins have distinct degradation rates and mechanisms of degradation. In some cases degradation is mediated by signaling events, whereby an initial signal orchestrates a chain of events resulting in protein turnover. In other cases, proteins are constitutively degraded in order to maintain cellular equilibrium (Inobe and Matouschek, 2014).

Several well-studied signaling networks involve the modulation of protein half-life as a key mechanism for mediating information flow. Given that many human diseases, including the pathogenesis of cancer, have defects in protein turnover (Nakayama and Nakayama, 2006), understanding the activities and levels of signaling proteins is crucial as they are critical determinants of how a cell will respond to a particular signal or environmental change.

The transcription factor NFκB is just one of the many proteins that requires constant regulation in order to maintain steady-state levels. Sustaining proper protein abundance can be done primarily by two degradation mechanisms namely, through the lysosome or through the proteasome. Lysosomal degradation is generally non-specific and the rate of degradation does not change upon stimulation (Ciechanover, 2005). In contrast, proteasomal degradation is highly specific and degradation rates can range on the timescale of minutes (like the enzyme ornithine decarboxylase; Asher et al., 2005)

to days (like the muscle proteins actin and myosin; Antecol et al., 1986). In the following section, we will focus on the turnover control by the proteasome.

PROTEIN TURNOVER CONTROL BY THE PROTEASOME

The proteasome is a large 700kDa protein complex that functions as a major piece of proteolytic machinery in higher eukaryotic cells. It is responsible for basal turnover of proteins in the cell, as well as the elimination of proteins with abnormal age or excess levels. However, the best-studied role of the proteasome is the spatial and temporal control of the destruction of many key cell regulators. In this aspect the proteasome occupies a central role in the control of cell division, differentiation, apoptosis, adaptation to stresses and, more generally, in the integration of most environmental signals (Figure 1.1.) (Coux et al., 1996).

The 20S core

The proteasome consists of two main parts: the proteolytic 20S core and the regulatory caps. The 20S core is made up of four heptameric rings stacked upon one another forming a central barrel. The two central rings are identical and are formed by seven β subunits each; the outer rings are also identical and are made up of seven α subunits each (Coux et al., 1996). The β rings contain three proteolytic sites on each ring: (1) a "chymotrypsin-like" site, which cleaves after large hydrophobic residues; (2) a "trypsin-like" site, which cleaves after basic residues; and (3) a "postglutamyl" hydrolase, which cleaves after acidic residues (Orlowski, 1990). The α rings on the other hand, have no proteolytic sites and instead seem to act as gatekeepers to the

opening of the proteolytic core. The 20S core interacts with three known regulatory caps, which are required to open the 20S core and are thought to have a role in recruitment and unfolding of substrates for hydrolysis.

The ubiquitin-proteasome system

The two most documented regulatory complexes are the 19S and 11S caps (Figure 1.2.; Ciechanover, 2005). The 19S regulatory particle is composed of nineteen subunits, which can be divided into two substructures; namely, the base and the lid (Lander et al., 2012). The lid is thought to be necessary for recognition of ubiquitylated proteins. Two 19S caps and a 20S core particle make up what is referred to as the 26S proteasome, which is perhaps the most-studied version of the proteasome. Most proteins are targeted to the 26S proteasome by poly-ubiquitin chains. Ubiquitin is a 76 amino acid protein that is typically covalently linked to lysine in a regulated multistep fashion but has also been shown to conjugate to cysteine, serine and threonine via thiodiester and hydroxyester linkages (Cadwell and Coscoy, 2005; Wang et al., 2007). Ubiquitin generally marks proteins for degradation by the 26S proteasome; however, proteins can also be susceptible to ubiquitin-independent degradation via the 20S core proteasome (Bercovich et al., 1989; Chen et al., 2007; Asher and Shaul, 2005).

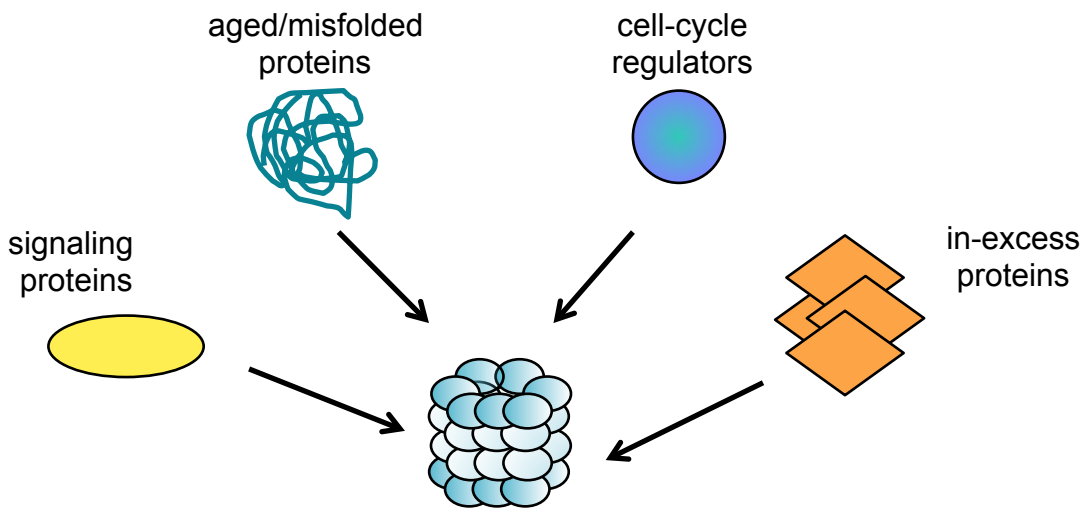


Figure 1.1. The physiological role of the proteasome.
The proteasome is responsible for basal turnover in the cell, as well as the elimination of abnormal, aged or in-excess proteins. Its most studied role is the spatial and temporal control of the destruction of many key cell regulators. The proteasome occupies a central role in the control of cell division, differentiation, apoptosis, adaptation to stresses and, more generally, in the integration of most environmental signals.

Ubiquitin-independent degradation

Although data remains inconclusive, it is thought that ubiquitin-independent degradation utilizes the other subset of regulator caps referred to as the 11S regulatory cap, which are also denoted as PA28 or REG complexes (Figure 1.2.). There are two types of 11S activators, which are either made of heteroheptamers of α and β subunits or of homoheptamers of γ subunits (Mao et al., 2008). The 11S regulators do not contain ATPase activity and can mediate proteasomal degradation independent of ATP and ubiquitin. Since the 19S cap has also been implicated in the degradation of proteins in the absence of ubiquitin (Jariel-Encontre et al., 2008), it is not clear whether all ubiquitin-independent degradation is mediated via the 11S cap.

There are an increasing number of signaling proteins that have been shown to be degraded by the proteasome without the requirement of prior ubiquitination including the proto-oncogene c-fos (Acquaviva et al., 2002), the genotoxic response regulator p53 (Asher and Shaul, 2005), and the polyamine synthesis enzyme ornithine decarboxylase (Asher et al., 2005). These proteins are regulated *via* modulation of their synthesis and degradation rates. In these systems, it is the protein itself that undergoes a high turnover rate. In contrast, the cellular abundance of the transcription factor NFκB does not change radically during signaling, but instead, the abundance of its inhibitor, IκBα, does (Hoffmann et al. 2002; O'Dea et al., 2007). NFκB is a critical mediator for cellular responses to inflammatory cytokines, developmental signals, pathogens, and cellular stresses (Hoffmann and Baltimore, 2006). While inflammatory signaling leads to short-lived NFκB activity that is carefully regulated

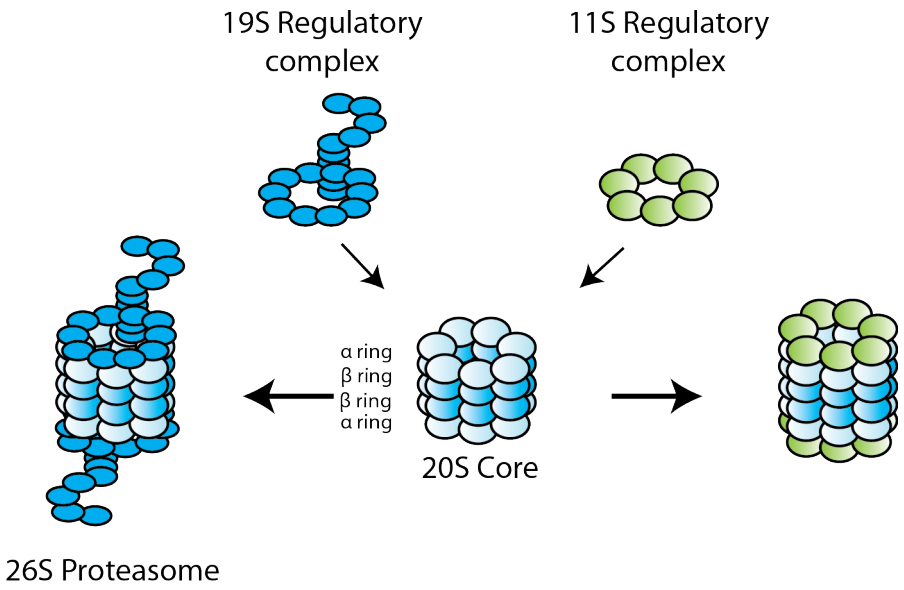


Figure 1.2. The components of the proteasome.
The 20S core is a barrel shaped cylinder composed of two inner β-rings and two outer α-rings. The α-rings mediate entrance to the proteolytic β-rings. There are at least two activators of the proteasome, the 19S and 11S caps.

via feedback mechanisms, constitutive high levels of active NFκB are associated with inflammatory diseases and many types of cancer (Courtois and Gilmore, 2006).

THE NFKB SIGNALING PATHWAY

The NFκB family of transcription factors are the effectors of a signaling system that is responsive to a large number of stimuli. These factors are mediated by most members of the tumor necrosis receptor (TNFR), the toll like receptor (TLR) superfamilies, as well as metabolic or genotoxic stress inducers (Figure 1.3.). As a critical regulator of immunity, NFκB signaling components can be found in almost all multi-cellular organisms, including mammals, insects, urchins, and mollusks but not *C. elegans* (Graef et al. 2001; Sullivan et al. 2007).

In mammals, the genes *rela, relb, crel, nfkb1,* and *nfkb2* respectively encode the five NFκB protein family members RelA (p65), RelB, c-Rel, p50, and p52. Thus forming homo- and heterodimeric DNA binding complexes (Figure 1.4. and Figure 1.5.). All five family members have a characteristic Rel homology domain (RHD) responsible for DNA binding and dimerization. However, while RelA, RelB, and c-Rel each possess a transcriptional activation domain (TAD), p50 and p52 do not. Thus, of the fifteen theoretically possible NFκB dimers, some function as transcriptional activators (notably the omnipresent RelA: p50 heterodimer), but others (such as the p50:p50 homodimer) do not unless they recruit specific co-activator proteins. Moreover, some dimers are not known to bind DNA at all (Figure 1.5.) (Hoffmann and Baltimore, 2006).

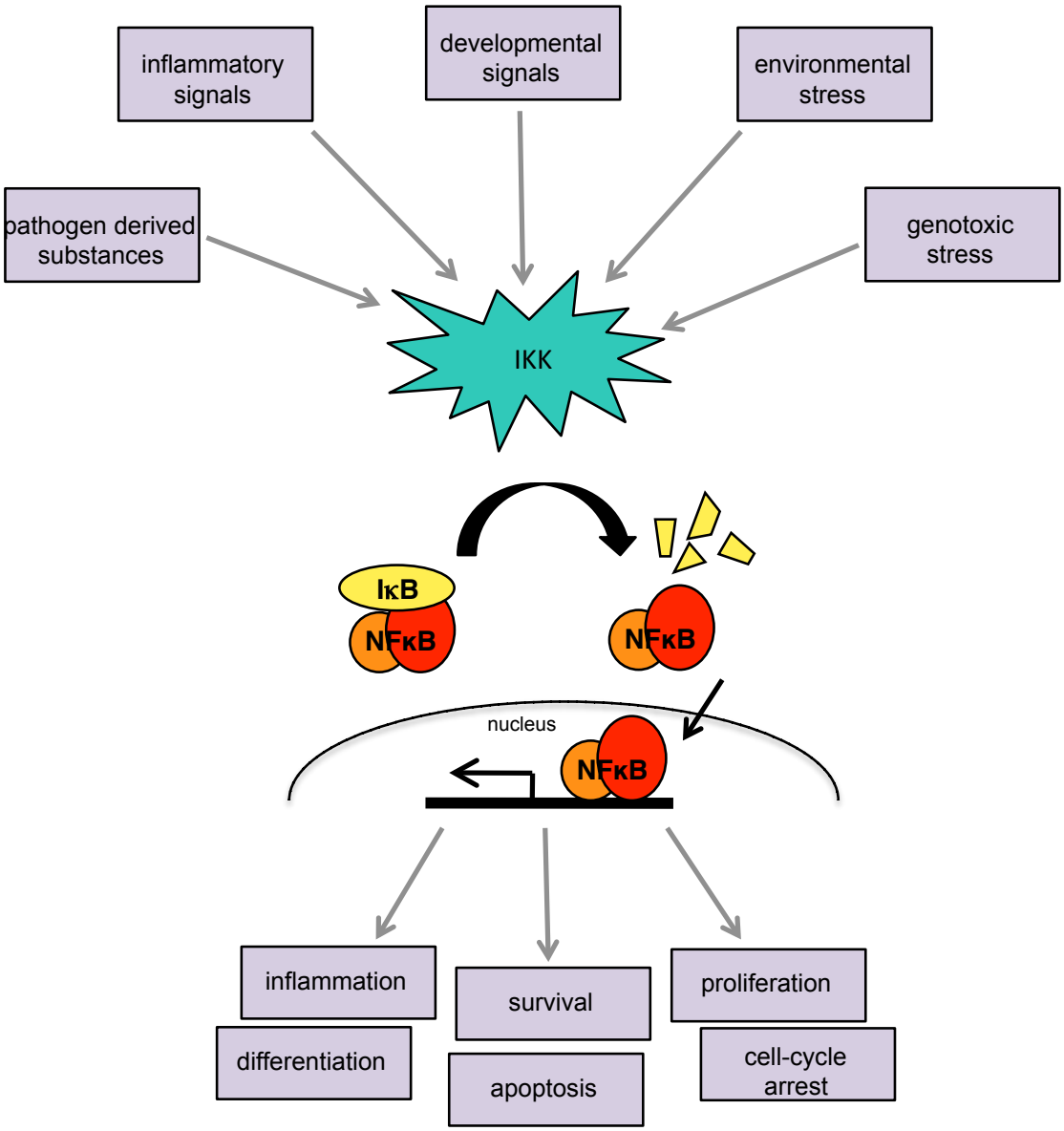


Figure 1.3. A schematic detailing NFκB activation and function in response to different types of signals.
Many different types of signals cause the activation of the IKK complex, driving the degradation of IκB and allowing for free NFκB to accumulate in the nucleus for activation of target genes. NFκB is the transcription factor responsible for driving the expression of a myriad of genes involved in different cell fates and physiological responses. This schematic was adapted from (Hoffmann 2006).

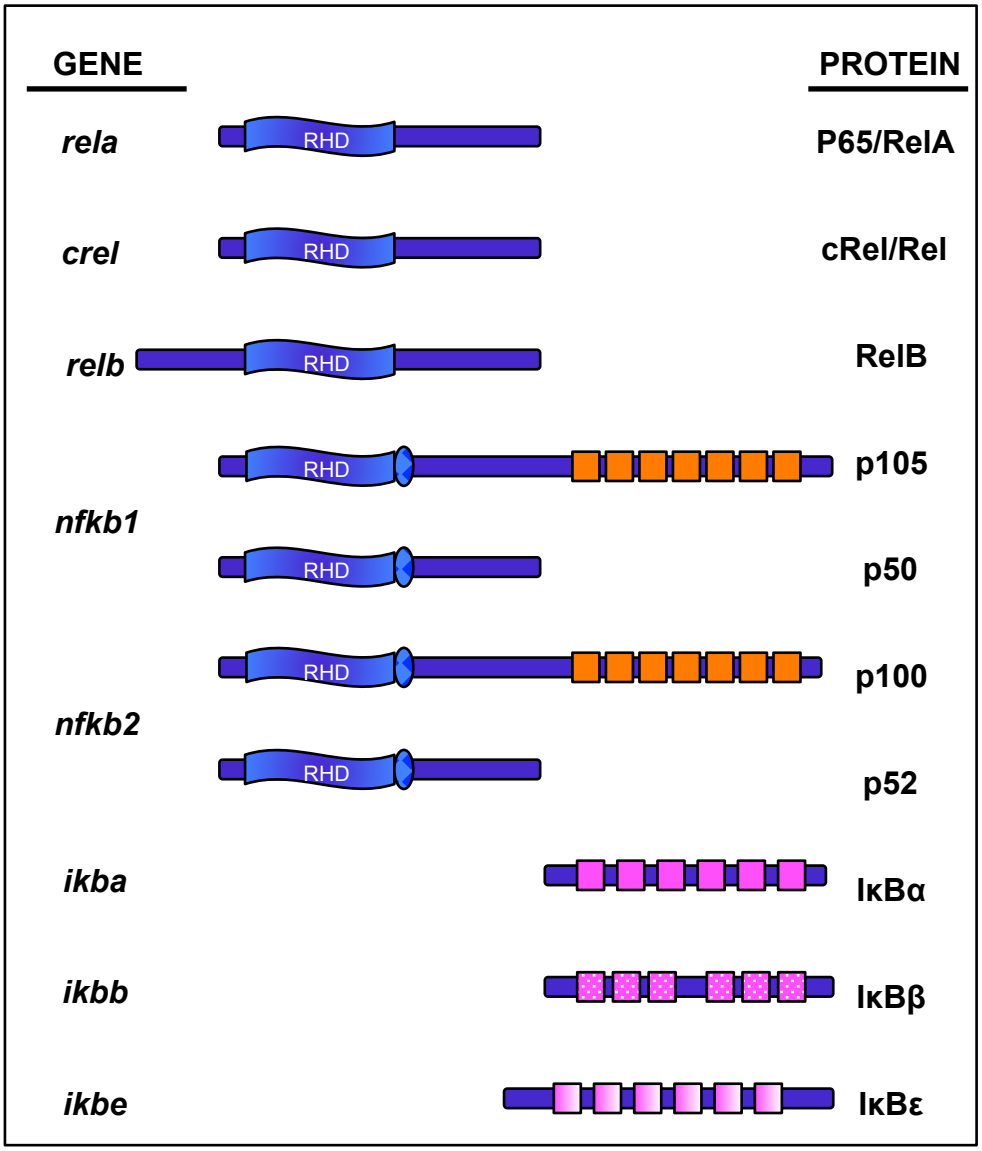


Figure 1.4. The NFκB and IκB family members.
Five mammalian NFκB genes give rise to five transcription factor proteins RelA, cRel, RelB, p50 and p52, that share the Rel-homology domain (RHD, blue banner), which is responsible for DNA-binding, dimerization, and association with IκB proteins. The proteins p50 and p52 are derived from a proteolytic processing event of the p105 and p100 precursor proteins, respectively. Their C-terminal portions contain ankyrin repeat domains (ARD) that are the hallmark of the classical IκB inhibitor proteins IκBα, IκBβ, and IκBε . This schematic was adapted from Hoffmann and Baltimore 2006.

In the absence of stimuli, NFκB dimers are retained in the cytosol through association with an Inhibitor of κB activity termed IκB. All IκB proteins contain a central ankyrin repeat domain (ARD), which is composed of six or seven copies of the consensus ankyrin repeat (Figure 1.4.). Ankyrin repeats (AR) are typically a 33 amino acid sequence motif that forms a consecutive β-hairpin-helix-loop-helix fold (Michaely and Bennett, 1993; Binz et al., 2003). The three classical or so-called 'canonical' IκB proteins, IκBα, IκBβ, and IκBε, are respectively encoded by the *nfkbia, nfkbib,* and *nfkbie* genes, and contain six ankyrin repeats in their ARD. Each IκB contains a signal responsive domain (SRD) that contains phosphorylation and ubiquitination sites for signal responsive degradation and IκBα and IκBβ also contain an acidic carboxy-terminal domain that is rich in the amino acids proline, glutamic acid, serine, and threonine (PEST). A fourth IκB was characterized, IκBδ, and results from the multimeric association of the *nfkb2*-encoded p52 precursor protein p100 (Basak et al., 2007). Together, these four IkBs prevent DNA binding by NFκB dimers and shift their cellular localization to the cytoplasm. A myriad of signals can cause the degradation of the IκBs, liberating NFκB from the canonical and non-canonical IκB proteins, and allowing translocation of NFκB and subsequent binding of κB site sequences (defined by a loose consensus of GGRNNN(N)YCC) in the promoters and enhancers of numerous genes (Hoffmann et al. 2003).

Other IκB-like proteins with different functions have also been identified. The p50 and p52 proteins are respectively produced via protein processing of the precursor proteins p105 and p100, each of which contains a C-terminal ARD (Figure 1.4.). Dimerization via the RHD of p100 or p105 with other NFκB proteins may result in

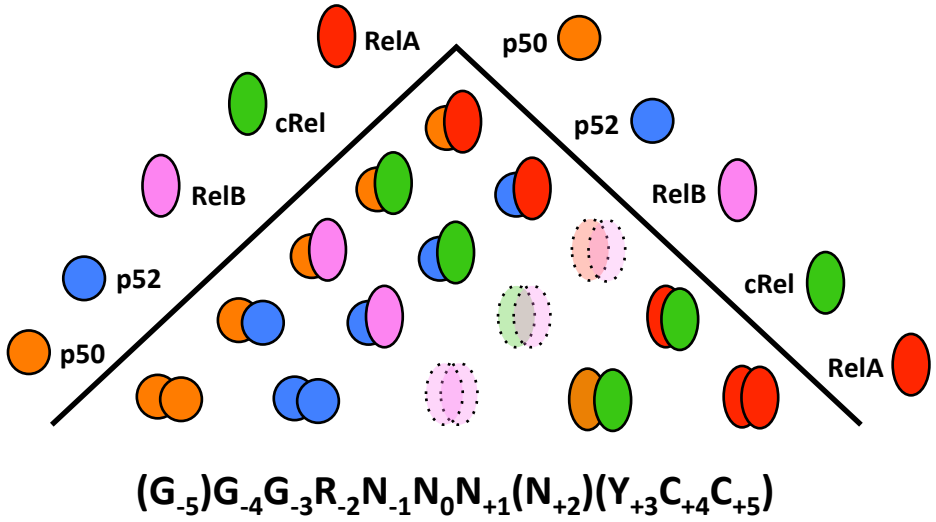


Figure 1.5. The NFκB homo- and heterodimers.
The five proteins can form 15 transcription factors via homo- or heterodimerization. While in principle, every isoform of one molecule type can interact with every member of the adjacent family, differential affinities make some interactions much more likely than others. In general, it is thought that the RelA:RelB, cRel:RelB and RelB:RelB dimers do not functionally bind DNA (denoted by transparent dimers). These dimers recognize a consensus κB site, as shown above. This schematic was adapted from Hoffmann et al., 2006

self-inhibited dimers. In addition, the IκB protein family members Bcl-3, IκBζ/MAIL, and IκBNS, bind subsets of NFκB dimers, but do not inhibit DNA binding. Instead these proteins may function as co-activators, like the TAD-deficient NFκB dimers p50:p50 and p52:p52 (Bundy and McKeithan 1997; Hirotani et al. 2005; Trinh et al. 2008; Yamamoto and Takeda, 2008, Vallabhrpurapu and Karin, 2009).

The canonical NFκB pathway

The canonical or "classical" NFκB pathway can be activated by a large number of stimuli; including: proinflammatory cytokines, bacterial lipopolysaccharide (LPS), and viral infections. Stimulation of cell surface receptors, such as the TNF receptor (TNFR), IL-1β receptor (IL-1βR), or toll-like receptors (TLRs), causes phosphorylation-dependent activation of the IκB kinase (IKK) complex. This multimeric complex is composed of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and the scaffolding protein, IKKγ (NEMO-NFκB essential modulator). Once activated, the canonical IKK complex phosphorylates IκBα, -β, or –ε at two specific serine residues. IκBα is the predominant IκB in most cells and is typically bound to RelA: p50. Phosphorylation of IκBα at ser^{32} and ser^{36} signals as a docking site for the E3 ubiquitin ligase β-TrCp, which catalyzes K48-linked ubiquitination at lysine 21 and 22 of IκBα, and leads to its subsequent degradation by the 26S proteasome (Hoffmann and Baltimore, 2006). Degradation of IκBα releases RelA: p50, allowing it to accumulate in the nucleus to bind DNA and activate gene expression (Figure 1.5). Several IκB proteins are among the large number of NFκB responsive genes, thus

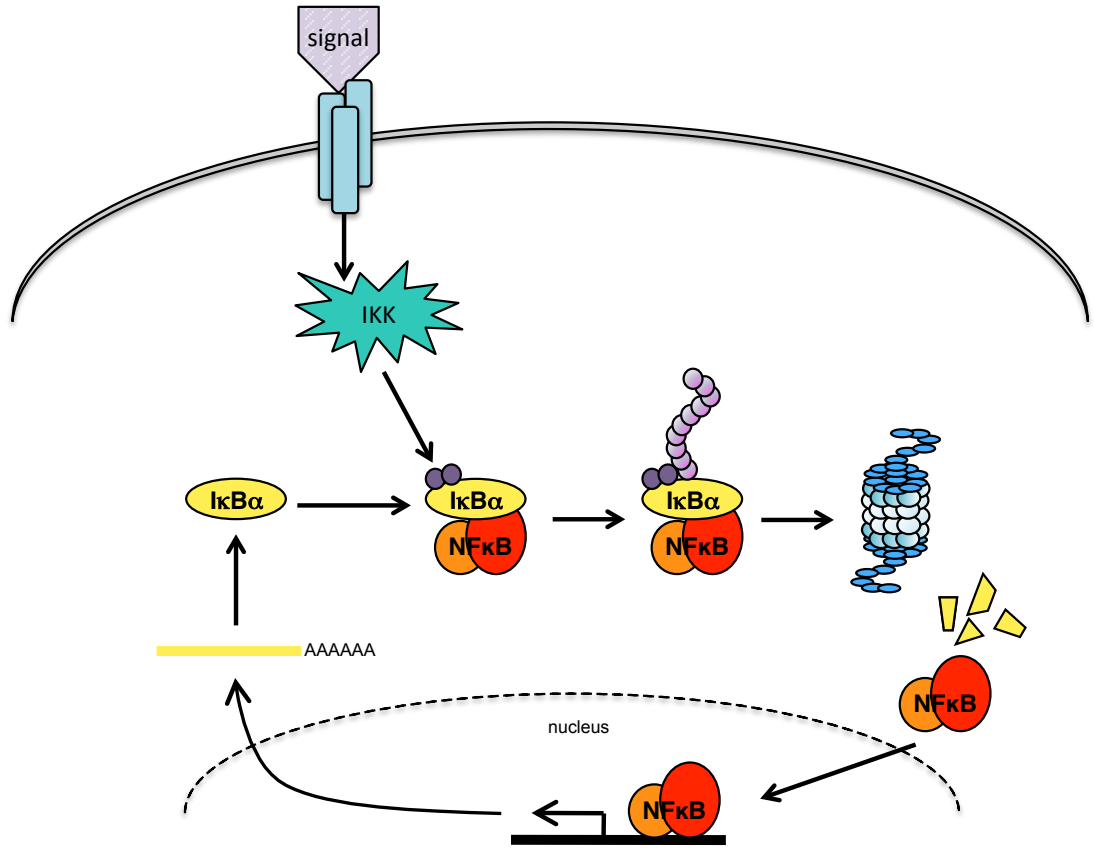


Figure 1.6. Canonical NFκB signaling pathway.
Canonical (inflammatory) signals activate IKK complexes (made of IKKα, IKKβ and NEMO), which target NFκB-bound IκBα (and β and ε) for degradation. IκBα (or β and ε) that is not bound to NFκB is constitutively degraded through an IKK-independent mechanism that does not rely on typical ubiquitin acceptors. Liberated NFκB (primarily RelA:p50 dimers) accumulates in the nucleus and activates gene expression, including the IκBα and IκBε genes.

regulating the rapid and transient NFκB-mediated response to cellular signals through NFκB induced negative feedback.

Free IκBα

In addition to the stimulus responsive IKK-dependent degradation of NFκB bound IκBα, there are reports that have shown the importance of unbound IκBα protein turnover (O'Dea et al., 2007; O'Dea et al., 2008; Mathes et al., 2008; Mathes et al., 2010). In resting cells approximately 85% of total cellular IκBα is bound to NFκB while 15% of IκBα is not in the IκB-NFκB complex (Rice and Ernst, 1993; Figure 1.7.). Furthermore, the *in vivo* half-life of free IκBα is about fifteen minutes (O'Dea et al., 2007; Mathes et al., 2008). Thus free IκBα is at least five-times more rapidly degraded in its basal state than when bound to NFκB (Pando and Verma, 2000).

As evidenced by the NFκB-IκBα crystal structure (Huxford et al., 1998; Jacobs and Harrison, 1998), when IκBα is bound to NFκB all six AR contact NFκB. In contrast, NMR spectroscopy has revealed that when free, the first four AR of IκBα are folded compactly whereas AR 5 and 6 are folded weakly and are highly flexible (Ferrerio et al., 2007; Truhlar et al., 2006; Croy et al., 2004). AR 5 and 6 adopt their fully folded conformation when bound to NFκB structure (Huxford et al., 1998; Jacobs and Harrison, 1998).

Synthesis and degradation of unbound IκBα is mostly constitutive and does not require IKK phosphorylation nor does it necessitate lysine-mediated ubiquitin conjugation (Mathes et al., 2008). Deletions in IκBα show that neither the N-terminal

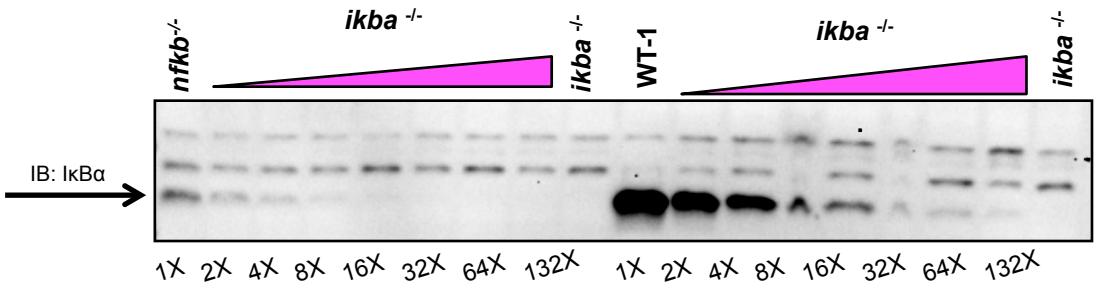


Figure 1.7. Free IκBα is 16X less abundant in *nfkb*$^{-/-}$ cells as compared to WT-1 cells. Immunoblot of IκBα in cells lacking RelA, c-Rel and p50, the canonical binding partners of IκBα. *nfkb*$^{-/-}$ cells and WT-1 cells. A serial dilution was done with *nfkb*$^{-/-}$ lysates to determine levels of free IκBα in the *nfkb*$^{-/-}$ cell line.

regions (Δ1-70) nor the C-terminal regions (Δ280-317) are required for free IκBα degradation *in vitro* by the 20S proteasome core (Krappmann et al., 1996; Alvarez-Castelaó and Castaño, 2005). In addition, through a kinetic based mathematical model, it has been shown that when the rapid degradation rate of IκBα is altered, NFκB activation is severely inhibited (O'Dea et al., 2007; O'Dea et al., 2008). These results indicate that the rapid protein turnover of IκBα is essential for buffering NFκB responsiveness to metabolic stresses.

FOCUS OF STUDY

It has been postulated that the degradation of free IκBα by the proteasome depends on the incompletely folded carboxy-terminal ankyrin repeat domains (Ferrerio et al., 2007; Truhlar et al., 2006; Croy et al., 2004). These repeats are fully folded when bound to NFκB (Huxford et al., 1998; Jacobs and Harrison, 1998), thus rendering IκBα resistant to the ubiquitin-independent degradation pathway. However, experimental evidence for this hypothesis has remained inconclusive. This dissertation has made use of biochemical techniques to further probe the degradation pathway of free IκBα. The work presented here has revealed that there is a specific amino-acid sequence, termed a degron, which targets IκBα (or heterologous proteins) to the proteasome. Interestingly, these amino acids are buried within the IκBα-NFκB complex, thus stabilizing IκBα, and allowing it to effectively inhibit NFκB. To supplement this new understanding of IκBα half-life control in the context of the other two canonical IκBs, we also investigated the half-life control of IκBβ and IκBε. I

show that while IκBβ may undergo similar control as IκBα, IκBε is in fact a stable protein suggesting that it is less suited to ensure NFκB inhibition in resting cells.

Chapter 2:

The Identification and Characterization of a Degron in IκBα

ABSTRACT

IκBα is the primary regulator of the immune and inflammatory response transcription factor NFκB. Early work has established that IκBα inhibition is controlled through NFκB-dependent synthesis and signal-responsive degradation via the ubiquitin-proteasome system (UPS). However, recent studies have shown that NFκB homeostasis and signaling also depend on unbound IκBα half-life control via IKK-independent proteasomal degradation. Here, we show that IκBα contains a ubiquitin-independent degron that may target the heterologous protein GFP for degradation via a proteasome-dependent, non-lysosomal pathway. We identify an eleven amino acid peptide residing within the non-standard ankyrin repeat 6 as the minimum sequence sufficient for conferring short half-life. These sequences are indeed required for IκBα's short half-life as a chimeric mutant containing analogous ankyrin repeat 3 sequences in place of the degron, is stable in cells.

INTRODUCTION

Protein turnover is critical for cellular homeostasis and response to environmental changes. Indeed, several well-studied signaling networks (e.g. p53, ß-catenin, and NFκB) involve control of protein half-life as a key mechanism for mediating information flow. Protein degradation is facilitated by either the lysosome, an organelle packed with enzymes for the breakdown of diverse biomolecules, or the proteasome, a 20S oligomeric molecular machine (Ciechanover et al,. 2005). Proteasomal protein degradation affords a high level of control of protein levels via a 19S regulatory cap that ensures that only proteins conjugated with K48 linked ubiquitin chains are in fact degraded (Ciechanover et al., 2005). The ubiquitin-proteasome system (UPS) may thus provide not only for homeostatic turnover control but also for rapidly induced degradation of targeted protein via the control of a specific ubiquitin ligase. The sequences within proteins that contain ubiquitin acceptors (e.g. lysines, threonines, serines), recruit ubiquitin ligases, and control half-life of proteins, have been termed degrons (Dohmen et al., 1994).

A number of proteins have been identified that undergo proteasomal degradation in the absence of ubiquitin (Jariel-Encontre et al., 2008; Orlowski et al., 2003). Such proteins are thought to contain degrons that target them to proteasomal degradation without the requirement of ubiquitin-ligase activity. For example, characterization of the degron in the enzyme ornithine decarboxylase revealed that it functions as a ubiquitin mimic, interacting with the regulatory cap (Godderz et al., 2011). However, in other cases, such as thymidylate synthase (Forsthoefel et al., 2004), Rpn4 (Ha et al., 2012), or IκBα (Mathes et al., 2008; Truhlar et al., 2008),

disordered regions of the protein have been implicated. In some cases the biochemical evidence suggests that the regulatory cap is dispensable altogether (Alvarez-Castelao and Castaño, 2005) such that the degron may confer targeting to the 20S enzymatic core complex of the proteasome. However, such degrons have not been characterized in detail and their existence remains controversial.

One of these intrinsically unstable proteins is IκBα, the key regulator of the transcription factor NFκB (Pando and Verma 2000). In resting cells, IκBα binds NFκB, covering NFκB's DNA binding surface, resulting in a predominantly cytoplasmic IκBα-NFκB complex (Baeuerle and Baltimore, 1996). Inflammatory stimuli trigger activation of the IκB kinases (IKK), which phosphorylate IκBα serine 32 and 36 within the signal responsive domain (SRD). Phosphorylation leads to ubiquitination on K21/22 and subsequent proteasomal degradation of IκBα, allowing freed NFκB to accumulate in the nucleus and activate transcription of a large number of genes (Ghosh et al., 1998).

However, unbound IκBα is degraded rapidly (Pando and Verma 2000; O'Dea et al., 2007; Mathes et al., 2008) and the stimulus-independent turnover of IκBα was shown to be important for NFκB homeostasis (O'Dea et al., 2007) and signal responsiveness (O'Dea et al., 2008, Loriaux et al., 2013). The molecular basis for the short half-life IκBα half-life is not well understood. The incompletely folded C-terminus of free IκBα was shown to be necessary for turnover (Mathes et al., 2008, Ferreiro et al., 2007, Truhlar et al., 2006, Croy et al., 2004). Also, mutations towards the ankyrin repeat consensus that increase its foldedness *in vitro* prolong IκBα half-life *in vivo* (Truhlar et al., 2008, Mathes et al., 2010). However, it remains unclear: (1)

whether the C-terminus contains a peptide sequence that is responsible for triggering degradation, confers a short half-life to heterologous proteins, and thus constitutes a degron; and (2) how such a "degron" relates to the folding mutants that appear to counteract its activity.

Here we report the identification of a degradation signal located in the C-terminal region of IκBα, which may be transferred to either the N- or C-terminus of the heterologous globular protein GFP. This degron mediates proteasomal-dependent but ubiquitin-independent degradation and is located in ankyrin repeat (AR) 6, encompassing an eleven amino acid sequence of this AR (IκBα 251-262). Furthermore, we show here that the degron of IκBα has a non-redundant function in the degradation pathway of the free protein.

RESULTS

IκBα contains a degron

Previous studies have established that IκBα has a short half-life in cells lacking NFκB proteins, whether IκBα is expressed from its endogenous gene or over-expressed with a retroviral expression vector (O'Dea et al., 2007; Mathes et al., 2008). To test whether high turnover could be transferred to a heterologous protein, we fused IκBα with the green fluorescent protein (GFP) within the retroviral expression construct. GFP was chosen as it is a long-lived, globular protein that is known to fold independently regardless of whether it is fused to other protein sequences (Zimmer et al., 2002). Following transduction of the NFκB-deficient *crel*$^{-/-}$*nfkb1*$^{-/-}$*rela*$^{-/-}$ 3T3 cell

line (hereafter referred to as *nfkb*$^{-/-}$) with the recombinant retrovirus encoding the fusion protein, we observed GFP fluorescence in the cytoplasm of a fraction of cells (Figure 2.1.). After thrity minutes of treatment with the translation inhibitor cycloheximide (CHX) this fluorescence disappeared. However, co-treatment with the proteasome inhibitor MG132 recovered the cytoplasmic fluorescence. Appearance of nuclear fluorescence was deemed to be background, as even untransduced cells treated with MG132 acquired strong nuclear fluorescence. These studies demonstrated that the short half-life control of IκBα could be transferred to the globular protein GFP and that proteolysis was sensitive to a proteasome inhibition.

There are two well-known intracellular protein degradation pathways in mammalian cells: the lysosomal pathway, which is generally non-selective (Levine et al., 2011); and the ubiquitin/proteasome system, which is selective via ubiquitin E3 ligases (Hershko and Ciechanover, 1998). Since free IκBα degradation can be rescued via the proteasome inhibitor MG132 (Mathes et al., 2008; Figure 2.1.), it has been assumed that free IκBα degradation occurs by way of the proteasome; however, the lysosomal pathway may also be inhibited by MG132 (Lee and Goldberg, 1998). We examined whether the lysosomal pathway may be involved by pre-treating IκBα-expressing *nfkb*$^{-/-}$ cells either with the lysosomal inhibitors, Bafilomycin-A (Baf-A) and Chloroquine (CQ) or with a proteasomal inhibitor MG132. We then administered cycloheximide (CHX) in a time course (Figure 2.2.). Only the MG132 pre-treatment condition leads to a rapid increase in protein levels, indicating that the degradation of free IκBα is mediated by the proteasome.

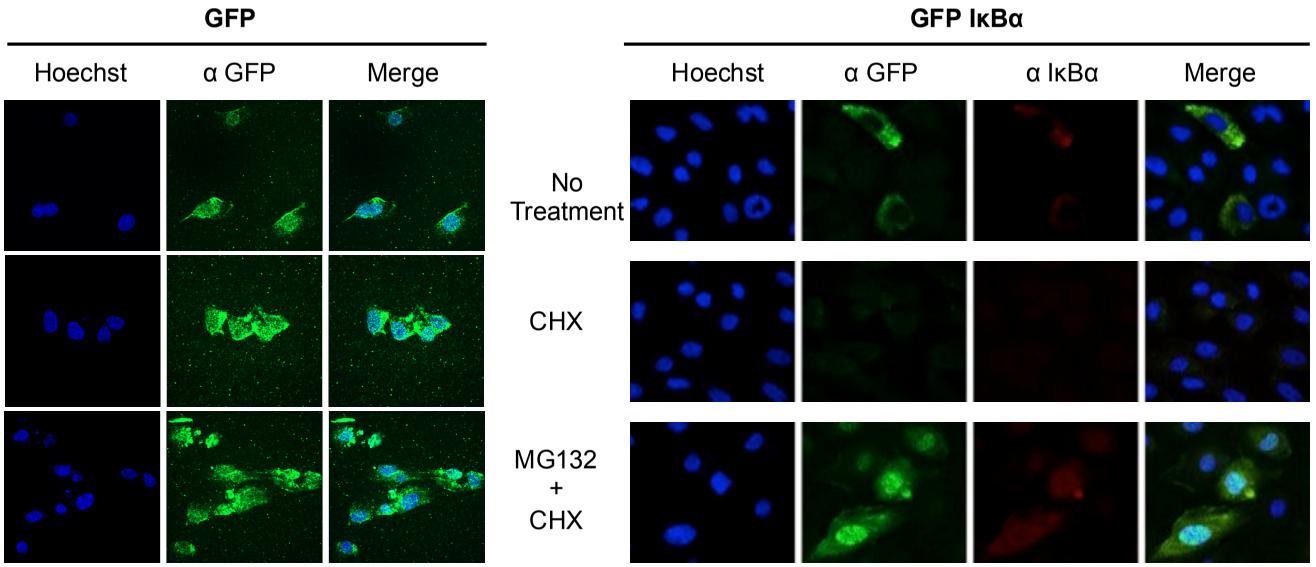


Figure 2.1. IκBα causes degradation of a heterologous protein.
Immunofluorescence for GFP and IκBα of *nfkb*$^{-/-}$ cells expressing GFP or GFP-IκBα, left untreated, treated with 10 μg/mL CHX for 30min, or pretreated with 10μM MG132 for 60min then treated with 10 μg/mL CHX for 30min.

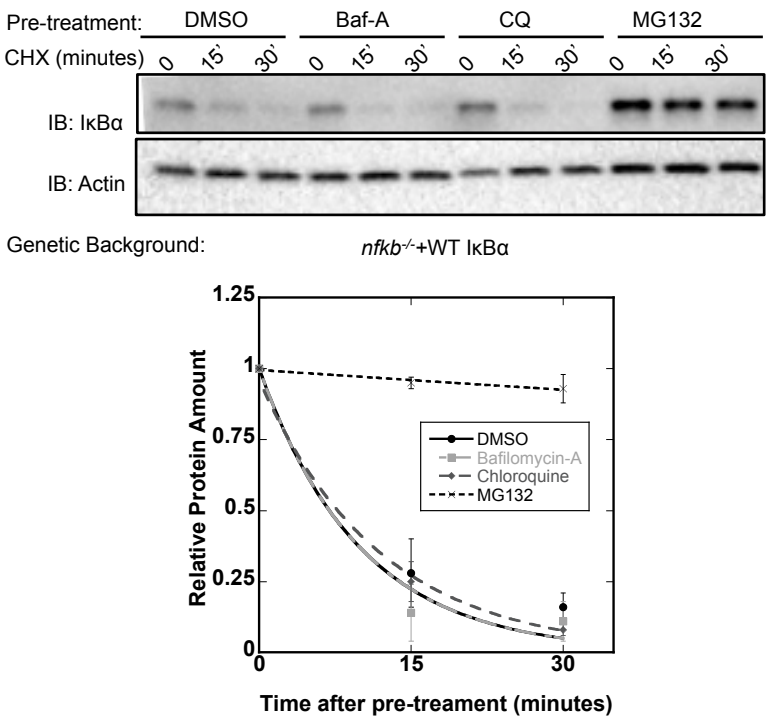


Figure 2.2. Lysosomal inhibitors do not extend free IκBα half-life.
Immunoblot for IκBα (C-terminal antibody) of whole cell extracts prepared from *nfkb*$^{-/-}$ cells stably expressing WT IκBα, pretreated for an hour with either DMSO, Bafilomycin-A, Chloroquine, or MG132 and then treated with CHX for indicated periods of time. The left panel shows quantification of 3 experiments, error bars indicate standard deviation

To identify the molecular determinants of IκBα's short half-life, we engineered a number of recombinant proteins using fragments of the IκBα protein. Figure 2.4. panel "a" depicts a schematic of IκBα's key structural elements and indicates the amino acid residue numbers that define the recombinant constructs designed. We first prepared recombinant retroviruses expressing full length human IκBα and a variant truncated after ankyrin repeat 4 at residue 206 and used them to infect *nfkb*$^{-/-}$ cells. The truncated form was expressed at a ten-fold higher level than the full-length form, and treatment with cycloheximide led to a rapid decrease of full-length, but not truncated IκBα levels (Figure 2.3.). Assuming an exponential decay relationship, we calculated a half-life estimate of around fifteen minutes for full length IκBα and of greater than 60 min for truncated IκBα. These results confirm previous conclusions that the C-terminus of IκBα, harboring ankyrin repeats 5 and 6 and the so-called PEST region, is required for rapid IκBα turnover (Mathes et al., 2008).

To determine whether this C-terminal portion of IκBα was a sufficient determinant for degradation, the C-terminal region, containing the 5th and 6th ankyrin repeat and PEST region, was fused to GFP and expressed in *nfkb*$^{-/-}$ cells. To measure the approximate half-life of the resulting GFP-IκBα(210-317) fusion protein, immunoblots for IκBα were performed in cells treated with cycloheximide. Whereas the GFP control construct was stable during the 60-minute time course, GFP-IκBα(210-317) showed a half-life of less than fifteen minutes (Figure 2.4. panel b). This indicates that the C-terminal region of IκBα is not only required but is also

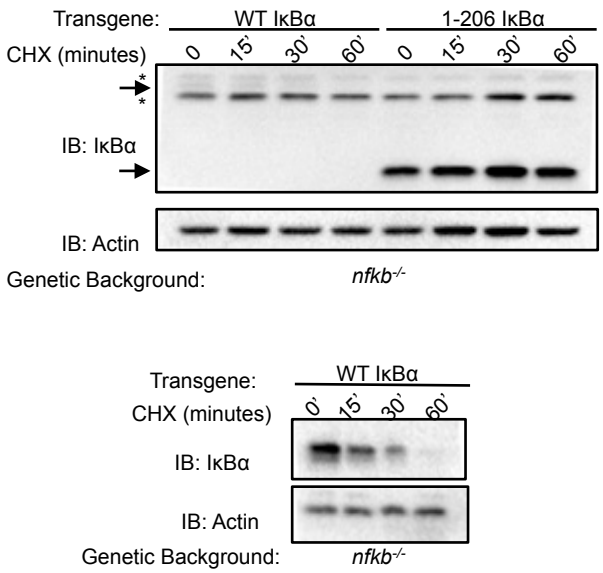


Figure 2.3. Deletion of IκBα C-terminus extends its half-life.
Immunoblots for IκBα (N-terminal antibody) of extracts prepared from *nfkb*$^{-/-}$ cells expressing WT IκBα or IκBα (1-206) treated with CHX. * Indicates nonspecific bands associated with this antibody. Lower panel shows same WT IκBα extracts probed with an antibody raised against the C-terminus of IκBα.

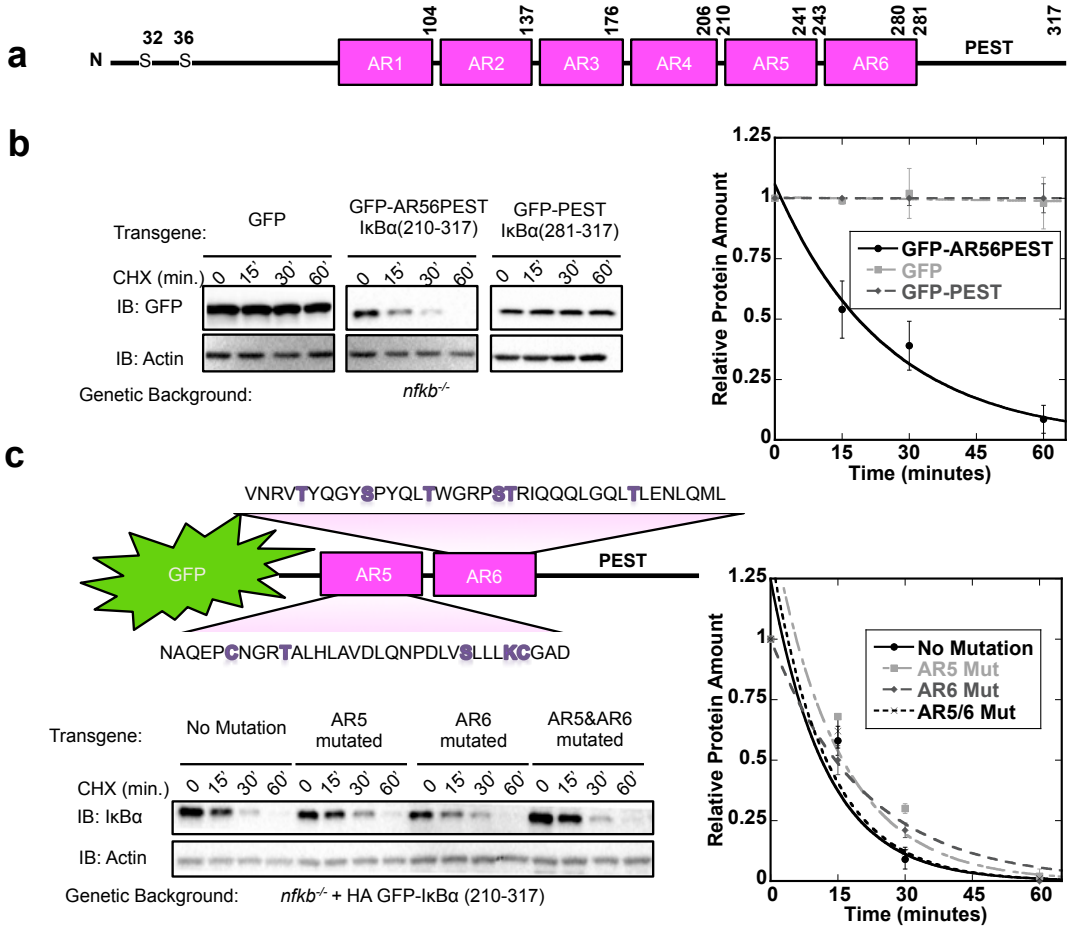


Figure 2.4. The C-terminal region of IκBα contains a degron.
Schematic of the IκBα protein. The signal response domain (SRD), the ankyrin repeat domains (ARD), and the PEST domain are indicated. IKK phosphorylation sites are indicated as well as the amino acid residues of the six ankyrin repeats (AR). (b) Immunoblot for IκBα of extracts prepared from CHX-treated *nfkb*$^{-/-}$ cells expressing GFP (GFP), GFP-IκBα (210-317) or GFP- IκBα (281-317). Right panel shows quantification of 3 experiments, error bars indicate standard deviation. (c) Upper panel: schematic of IκBα indicating the amino acids in the 5th and 6th repeats, and atypical ubiquitin acceptors are highlighted. Lower left panel shows *nfkb*$^{-/-}$ cells expressing either non-mutated IκBα(210-317), IκBα(210-317) where all ubiquitin acceptors are mutated within AR5, within AR6, or within both AR5 and AR6 were treated with CHX and protein levels were detected by Western blot using an antibody directed against the C-terminus of IκBα. Lower right panel shows quantification of experiments, error bars indicate standard deviation.

sufficient to cause rapid protein turnover, suggesting that the C-terminal region of IκBα contains a degron. Previously the PEST sequence had been reported to play a role in the high turnover of proteins (Rogers et al. 1986). However, we found that the PEST domain of IκBα was not sufficient to grant degradation and in fact was stable during a 60-minute time course (Figure 2.4. panel b).

It was previously shown that the lysine residues of free IκBα are not required for free IκBα degradation (Mathes et al., 2008). However, ubiquitin conjugation on other amino acids such as cysteine, serine, and threonine via thiodiester and hydroxyester linkages has been reported to mediate proteasomal degradation (Cadwell et al., 2005, Wang et al., 2007). To test whether these atypical ubiquitin acceptors might play a role in the degradation of IκBα, we mutated five residues in ankyrin repeat 5 and six residues in ankyrin repeat 6 to alanine, with the exception of lysine, which was mutated to arginine. Following retroviral transduction into *nfkb*$^{-/-}$ cells, cycloheximide time courses revealed no change in the half-life of the mutants (Figure 2.4. panel c). These results indicate that the IκBα degron targets proteins for degradation by the proteasome via a ubiquitin-independent, non-UPS mechanism.

Interestingly the constitutive and nuclear PA28γ, a proteasome activator that forms homomeric 20S proteasome caps, was shown to mediate ubiquitin-independent proteasomal degradation of the cell cycle regulators p21, p16, and p19, as well as the oncogene SRC-3 (Li et al. 2006; Chen et al. 2007; Li et al. 2007). To establish whether the PA28 proteins had an effect on free IκBα degradation, we used immortalized 3T3, cells deficient in all three PA28 proteins (deficient in PA28α, -β, and –γ), and transduced GFP-IκBα(210-317). Following CHX time courses, no

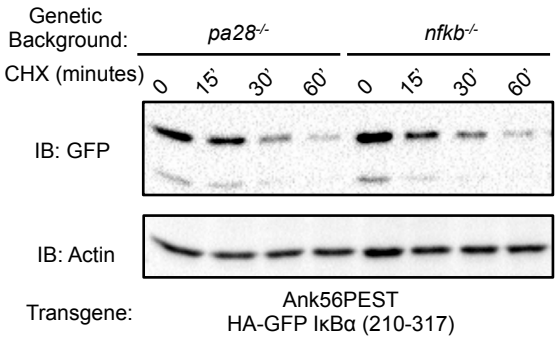
noticeable difference in the degradation rates of GFP-IκBα(210-317) in *pa28*$^{-/-}$ cells as compared to *nfkb*$^{-/-}$ cells was detected (Figure 2.5. panel a). As PA28γ is largely nuclear, the cytoplasmic and nuclear fractions of the transduced *nfkb*$^{-/-}$ cells were analyzed in order to investigate whether there was an effect on degradation in either cellular location. This approach revealed that no changes in protein levels could be detected, indicating that the PA28 proteins are not required in MEFs. However, it is possible that over expression of the proteasome activator proteins could lead to degradation of IκBα in wild-type cells.

The degron resides in the first half of ankyrin repeat 6

To identify the peptide sequence conferring degradation, we prepared constructs of the isolated 5th and 6th ankyrin repeat (AR5 and AR6) using the GFP-fusion expressing *nfkb*$^{-/-}$ cell system. In cycloheximide time courses (Figure 2.6. panel a), GFP-IκBα (AR5: 210-241) showed very similar degradation kinetics to the stable GFP control and PEST domain of IκBα (Figure 2.4 panel b). However, the GFP-IκBα (AR6: 243-280) construct showed rapid degradation analogous to the degradation of the complete C-terminus IκBα (281-317). The quantitated data shows that the half-life of the GFP-IκBα (281-317) as well as the GFP-IκBα (210-241) have half lives greater than two hours while the 6th ankyrin repeat have a half-life of around fifteen minutes. These findings indicate that the degron of IκBα is in its 6th ankyrin repeat.

Previous work established that the short half-life of thymidine synthase (TS) depends on a ubiquitin-independent degron, which must be located at the end of the N-

a



b

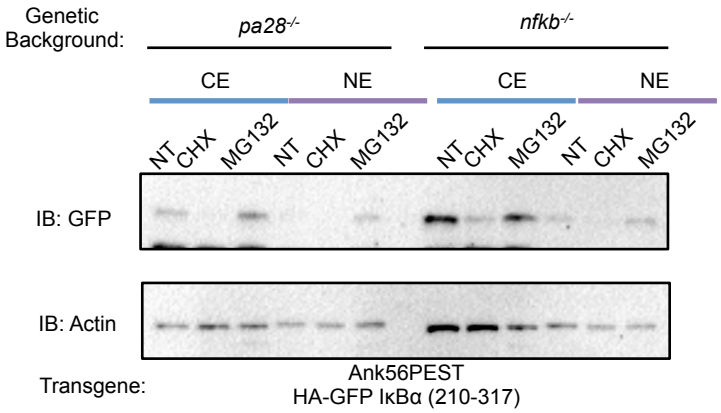


Figure 2.5. Deletions of the proteasome activators, PA28, have no effect on free IκBα degradation.
Immunoblot for GFP in *pa28*$^{-/-}$ or *nfkb*$^{-/-}$ cells transduced with HA-GFP IκBα (210-317) after treatment with CHX. (b) Cytoplasmic and nuclear extracts of *pa28*$^{-/-}$ or *nfkb*$^{-/-}$ cells transduced with HA-GFP IκBα (210-317) were treated with either CHX or MG132 and then probed with GFP.

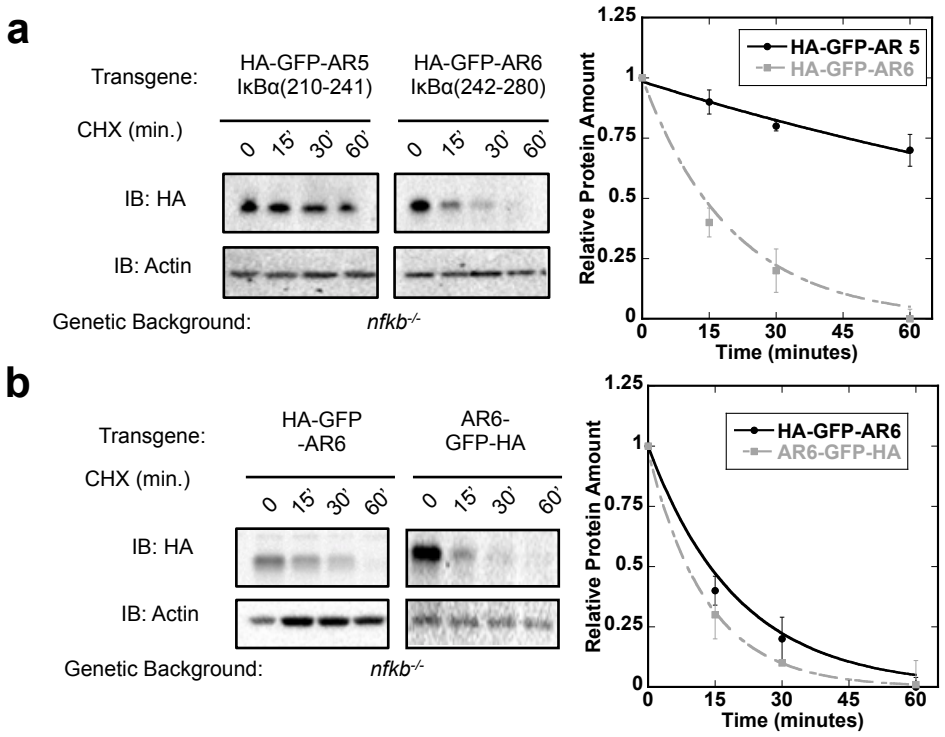


Figure 2.6. The degron resides in ankyrin repeat 6.
Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR5 (IκBα (210-241)) or AR6 (IκBα (242-280)) treated with CHX. Right panel shows quantification of experiments, error bars indicate standard deviation and are representative of at least 3 experiments. (B) Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR6 (IκBα (242-280)) with the HA-GFP tag at either the N- or C-terminus of the protein, treated with CHX. Right panel shows quantification of experiments, error bars indicate standard deviation and are representative of at least 3 experiments.

terminus; blocking the N-terminus with a His-tag caused protein stabilization (Peña et al., 2006). To examine whether the IκBα degron depends on its C-terminal location, we engineered a construct in which the HA-GFP tag was fused to the carboxy rather than amino terminus of the 243-280 region (Figure 2.6. panel b). Cycloheximide time course data show that the degron's location does not affect its activity.

The C-terminal region of IκBα has been shown to be incompletely folded when not bound to NFκB (Ferrerio et al., 2007; Truhlar et al., 2006; Croy et al., 2004). It is possible that the internal ankyrin repeats AR2 and AR3 may also be harboring degrons as well, whose activities are hidden within the folded ARD of AR1-4. As three ARs are required for stable folding of an ARD (Michaely and Bennett, 1993), we examined AR2 and AR3 in isolation to reveal any latent degron activity. Strikingly, constructs containing the fragments of either AR2 or AR3 did not cause degradation of GFP after 60-minute exposure to CHX (Figure 2.7). These data indicate that there are specific degron sequences within AR6 that are not present in other ankyrin repeats of IκBα.

To determine which segment of AR6 determines free IκBα degradation rate, we explored the functional differences between the structurally homologous AR3 and AR6 to design a series of chimeric constructs (Figure 2.7. panel a). First, we fused the first half of ankyrin repeat 6 to the second half of ankyrin repeat 3 (mutant 6633), as well as its inverse (mutant 3366). Within the *nfkb*$^{-/-}$ cell system the 3366 mutant showed a half-life greater than 60 minutes while the 6633 mutant had a half-life similar to the full ankyrin repeat 6 (Figure 2.7. panel b). These observations lead us to conclude that the first half of ankyrin repeat 6 (IκBα 243-262) contains the IκBα degron.

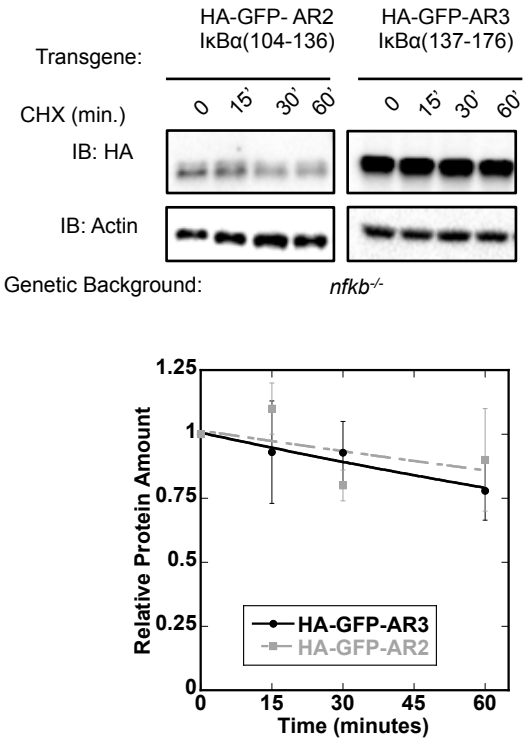


Figure 2.7. Other ankyrin repeats do not contain degrons.
Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR3 (IκBα (137-176)) or the AR2 (IκBα (104-136)) treated with CHX. Right panel shows quantification of experiments, error bars indicate standard deviation and are representative of at least 3 experiments.

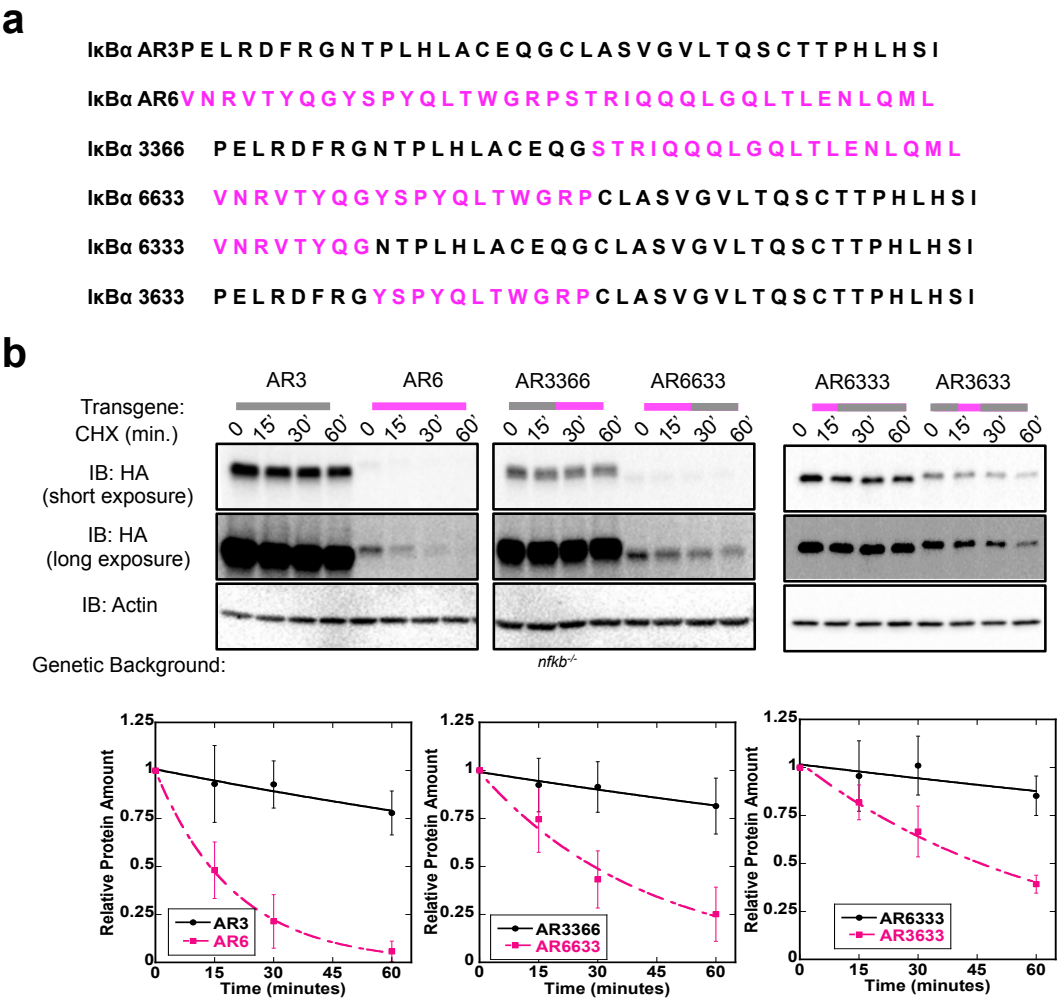


Figure 2.8. The first half of ankyrin repeat 6 contains the degron.
(a)Schematic detailing the amino acids in AR3 (in grey) and AR6 (in pink). The 4 chimeras (3366, 6633, 3633, 6333) contain the indicated amino acids derived from the color-coded AR. (b) Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing the 4 chimeras treated with CHX. Bottom panel shows quantification of experiments, error bars indicate standard deviation and are representative of at least 3 experiments.

In order to further probe the amino acid sequence responsible for IκBα degradation, we made two more chimeric proteins whereby either the first quarter of ankyrin repeat 6 was fused to ankyrin repeat 3 (mutant 6333) or the second quarter was fused into ankyrin repeat 3 (mutant 3633, Figure 2.8. panel a). Within the *nfkb*$^{-/-}$ cell system we observed a relatively short half-life for the 3633 mutant (Figure 2.8. panel b) compared to the more stable 6333 mutant. T hese results indicate that the IκBα degron is located within the region of residues 251-262. As the 3633 mutant does not show as short a half-life as the full length AR6, we conclude that additional residues within AR6 may contribute to the degron's activity.

Further inspection of AR6366 and AR6333 demonstrates that tyrosine 254 and threonine 257 were present in the region of AR6 that we had suspected to be responsible for degradation (Figure 2.9. panel a). Mutations of these residues had previously been reported to increase IκBα's foldedness *in vitro*, thus prolonging its half-life *in vivo* (Truhlar et al., 2008, Mathes et al., 2010). We investigated whether the YLTA mutation (whereby Y254 was mutated to leucine and T257 was mutated to alanine) in IκBα (210-317) would render it more stable. After careful investigation, we came to the conclusion that while mutations in these residues lead to increased foldedness and decreased activity in the context of the full protein, the degron's activity does not rely on either Y254 or T257 (Figure 2.9. panel b).

Our studies thus far established that the first half of ankyrin repeat is sufficient for triggering degradation of the heterologous reporter protein GFP. Next, we sought

a

```
ank3__1-19_ank6  PELRDFRGNTPLHLACEQGSTRIQQQLGQ-LTLENLQML
ank6_1-19_ank3_  VNRVTYQGYSPYQLTWGRPCLASVGVLTQSCTTPHLHSI
consensus        -e---frG-tP-hLa--q-s------L-Qs-T--nLq-l
```

b

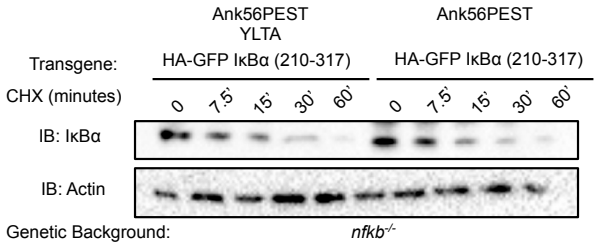


Figure 2.9. The degron does not rely on Y254 or T257.
(a) Alignment of AR3366 with AR6633, alignment was done using ClustalW and Boxshade. Green indicates fully conserved residues and cyan indicates semi-conserved residues. The red line is indicative of the quarter constructs. (b) Immunoblot for IκBα of whole cell extracts from *nfkb*$^{-/-}$ cells transduced with either IκBα (210-317) YLTA (whereby Y254 was mutated to leucine and T257 was mutated to alanine) or IκBα (210-317) treated with CHX for indicated times.

to determine whether this sequence might also be required for IκBα degradation in the context of the full-length protein. We engineered a mutant of IκBα that had its 6th ankyrin repeat replaced with the 3366 chimeric repeat (36M IκBα, Figure 2.10. panel a); stably transduced this construct, as well as wild-type IκBα and the long lived IκBα(1-206) into *nfkb*$^{-/-}$ cells, and performed cycloheximide time courses. Immunoblotting revealed a stabilization of IκBα in the 36M that was similar to the IκBα(1-206) control (Figure 2.10. panel b). These results establish that the first half of the ankyrin repeat 6 contains a degron sequence that is both sufficient for degrading heterologous proteins and required for the degradation of free IκBα. We conclude that the degron of IκBα has a non-redundant function in IκBα degradation.

DISCUSSION

This study has identified and characterized the ubiquitin-independent, proteasome-dependent degradation signal of IκBα. We have shown that the degron is located in the 6th ankyrin repeat of IκBα and that it is sufficient to cause degradation of a heterologous protein, GFP. Interestingly, we find that the PEST region of IκBα on its own is not a sufficient degradation signal for IκBα. This negatively charged region of IκBα has long been thought to follow the PEST hypothesis, which posits that the PEST sequence is responsible for protein turnover (Rogers et al., 1986). While it is possible that the serine and proline rich region of IκBα may be important for other functions, we have shown here that this region does not contain the signal for

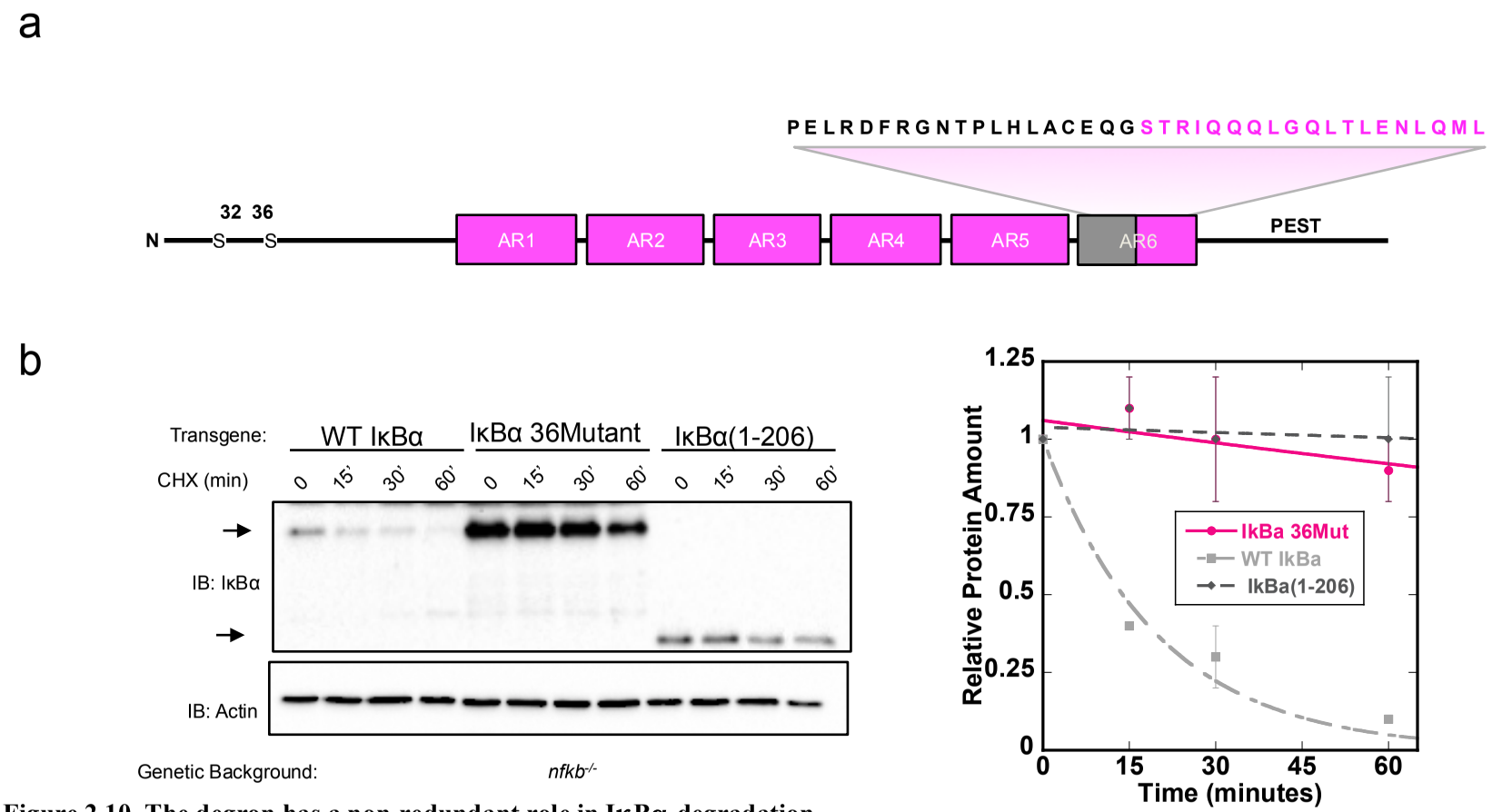


Figure 2.10. The degron has a non-redundant role in IκBα degradation.
(A) Schematic of the 36M IκBα. The amino acid sequence of the mutant is highlighted, the grey letters indicate AR3 residues and the pink letters indicate AR6 residues. (B) Immunoblot for the N-terminus of IκBα of *nfkb*$^{-/-}$ cells expressing WT IκBα, IκBα 36M, or IκBα(1-206) treated with CHX. Right panel shows quantifications of at least 3 experiments, error bars indicate standard deviation.

constitutive degradation of IκBα. We have shown that the degron is unique and does not appear to rely on the incompletely folded region of IκBα, but instead relies on specific amino acid residues within ankyrin repeat 6 that are hidden when properly folded. There have been reports of 20S proteasomal cores requiring a highly conserved tyrosine residue in their activation particles, which are required for gate opening (Dange et al., 2011; Yu et al., 2010; Rabl et al., 2008). Based on these reports, we hypothesize that Y251, Y254, or W258 might have direct contact with the 20S proteasome core, which has been shown to be sufficient to cause degradation of free IκBα in vitro (Alvarez-Castelao and Castaño, 2005; Mathes et al., 2008).

Our findings provide insight into the emerging field of ubiquitin-independent degradation. The ubiquitin proteasome system has been well studied and the degradation signal for the proteasome has traditionally been classified as ubiquitin chains regulated by E3 ligases. The current understanding of ubiquitin-independent protein degradation suggests that the lack of folding stability of a protein might be one pathway by which it can be targeted for degradation irrespective of ubiquitin conjugation (Jariel-Encontre et al., 2008). IκBα, when not bound to NFκB, has an incompletely folded C-terminus (Ferrerio et al., 2007; Truhlar et al., 2006; Croy et al., 2004), suggesting that its constitutive degradation may occur in such a fashion. It is evident from the present work that lack of foldedness is not sufficient, but rather specific sequences must exist to target the protein to the proteasome. However, IκBα degron activity appears to depend on being solvent exposed, and thus conditional on the protein being not bound to NFκB. Interactions with NFκB and NFκB-triggered folding of the C-terminus dramatically diminish IκBα degron activity. Indeed, the

eleven amino acid peptide sequence is situated in close proximity with the NFκB interaction surface in the crystal structure of the IκB-NFκB complex (Huxford et al., 1998; Figure 2.11.). The effect that an unregulated degron has on NFκB activity remains to be investigated.

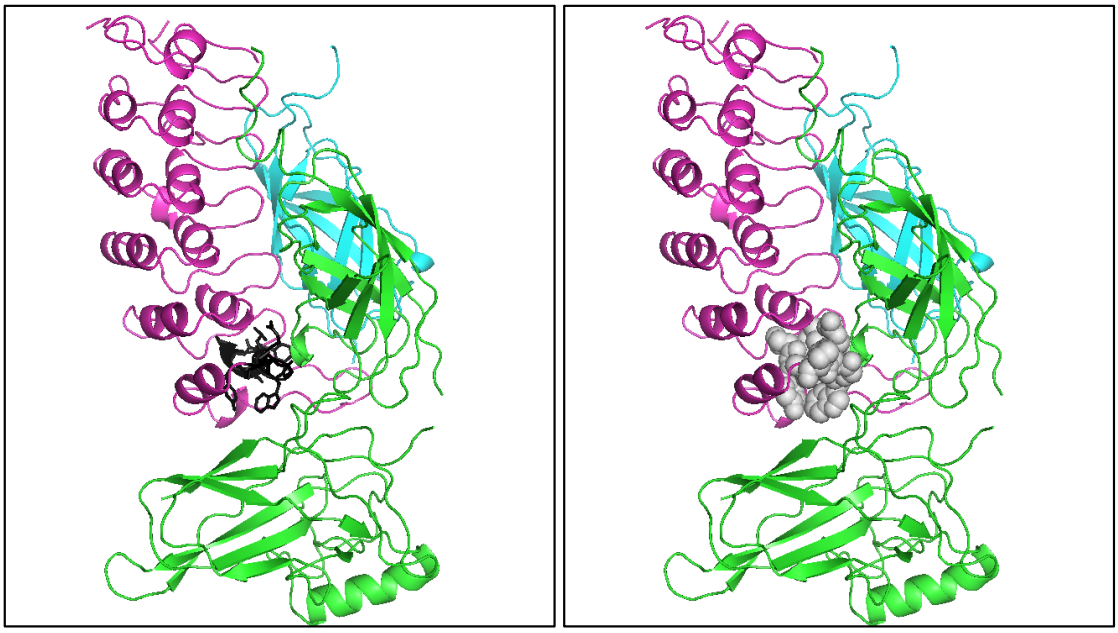


Figure 2.11. Location of the degron in the IκBα-NFκB complex.
A ribbon diagram representation of the crystal structure of IκBα (pink) bound to NFκB (p50, cyan; p65, green). Left panel shows the location of the 11 amino acid degron in black, depicted with ball-and-stick representation. Right panel shows the location of the 11 amino acid degron in space fill representation and colored in grey. The figure was prepared using the PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC.

MATERIALS AND METHODS

DNA constructs.

IκBα constructs were cloned into the retrovirus vector pBabe-puro between the restriction sites EcoRI and SalI. HA-GFP was cloned between the sites BamHI and EcoRI. Mutations were made using standard polymerase chain reaction-based methods.

Cell Culture.

Immortalized 3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum, 1% L-glutamine, and 1% penicillin and streptomycin at 5% CO_2, 37^{o}C. Cells treated with cycloheximide (Calbiochem) were used at 10 μg/mL in 100% EtOH. For proteasome inhibition, 10μM MG132 (Sigma) was used. For virus production, Plat-e cells (Morita et al., 2002) were transiently transfected with PEI (Reed et al., 2006) with 10 μg of pBabe and allowed to grow for 48 hours. Filtered virus was placed onto target cells along with 4μg/mL Polybrene (Sigma). Infected cells were selected with 2.5-μg/mL puromycin (Sigma).

Immunofluorescence.

Mouse 3T3 cells were grown on glass coverslips (Fisher) and fixed in 4% PFA (EM Sciences) for 10 minutes at RT. Slides were blocked with 5% Normal Goat Serum, 0.2% Triton-X100 in PBS and stained with primary antibodies in blocking buffer: sc-996 mouse-anti-GFP at 1:200 dilution and sc-371 rabbit-anti-IκBα at 1:200 dilution for overnight at 4°C. Secondary antibodies used were goat-anti-mouse Alexafluor-488 (Life Technologies), 1:1000 and goat-anti-rabbit Alexafluor-568 (Life Technologies), 1:1000 at RT for 1hr. Images were acquired on an Axio Observer Z1

inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 40x, 1.3 NA oil immersion objective to an Orca Flash SCMOS camera (Hamamatsu, Japan) using ZEN imaging software (Carl Zeiss Microscopy GmbH, Germany).

Biochemical Assays.

After treatment with cycloheximide, cells were lysed in RIPA buffer. Cell extract was separated on a 4-15% gradient SDS-PAGE and transferred to PVDF membrane. IκBα was probed with either sc-371 (Santa Cruz Biotechnology) or sc-203, GFP was probed with sc-996 and HA was probed with 16b12 (Covance) then followed by HRP conjugate. Cycloheximide and MG132 was from Sigma. Quantification of immunoblots was performed with ImageJ. Dilution series with knockout extracts assured that Western blot signals were in the linear range.

Software.

Alignments were performed using ClustalW and Boxshade via the San Diego Super Computer Biology Workbench. Cartoon diagrams of IκBα were prepared using PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC using PDB file 1IKN

ACKNOWLEDGEMENTS

Jesse Vargas showed the dissertation author how to perform immunofluorescence and provided guidance in imaging. Russell Lewis and myself performed the experiments. This chapter in its entirety, except for the PA28KO work (Figure 2.5) has been submitted for publication in which R. Lewis will have authorship and Alexander Hoffmann will be the corresponding author.

Chapter 3:

Characterization of the Function of the IκB Degron in NFκB Signaling

ABSTRACT

Whereas ubiquitin-dependent degrons have been well characterized, the notion of a ubiquitin-independent degron remains controversial. In Chapter 2, we showed that IκBα contains a ubiquitin-independent degron that confers a short half-life control to the heterologous protein GFP. Using a combined biochemical, synthetic-biology, and computational approach, we demonstrate that while the IκBα degron is required for constitutive free IκB turnover. Furthermore, its activity must be regulated by protein-interaction-catalyzed folding, rendering IκBα more stable in cells when complexed with NFκB. Thus, we provide evidence for the existence of ubiquitin-independent degrons and reveal that their activity can be regulated not by enzyme-mediated linkages of ubiquitin, but by protein-protein interactions.

INTRODUCTION

NFκB is a transcription factor that has major roles in the immune system. It is a critical mediator of the development and maturation of immune organs and is involved in the rapid activation of the immune response, both which occur at very different timescales. The canonical pathway (associated with inflammatory stimuli) of NFκB functions on a relatively short time scale, of minutes-to-hours, and is controlled by NEMO-dependent kinase activity (Hoffmann and Baltimore, 2006). Conversely, the non-canonical pathway (associated with development of immune organs) operates on a long timescale, of hours-to-days, and is controlled by NEMO-independent kinase activity (Hoffmann and Baltimore, 2006). In mammalian cells, the NFκB transcription factors can exist as homo- or heterodimers of the NFκB family members RelA (p65), c-Rel, RelB, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100) (Figure 1.4. and 1.5.). The RelA: p50 complex is considered the principal dimer responsible for NFκB activity in most cell types (Vallabhapurapu and Karin, 2009).

NFκB is held inactive in the cytoplasm through stoichiometric association with inhibitory proteins (IκBs) (Karin and Ben-Neriah, 2000; Hoffmann and Baltimore, 2006). IκBα, IκBβ, and IκBε form the classical IκB family. In addition to the classical IκB family members there are atypical IκBs, which include IκBγ, IκBδ, and the additional NFκB interacting proteins: Bcl-3, IκBζ, and IκBNS (Vallabhrpurapu and Karin, 2009). IκBs have characteristic ankyrin repeat domians (ARD) that interact with NFκB. Although they are similar in structure, they have preferential

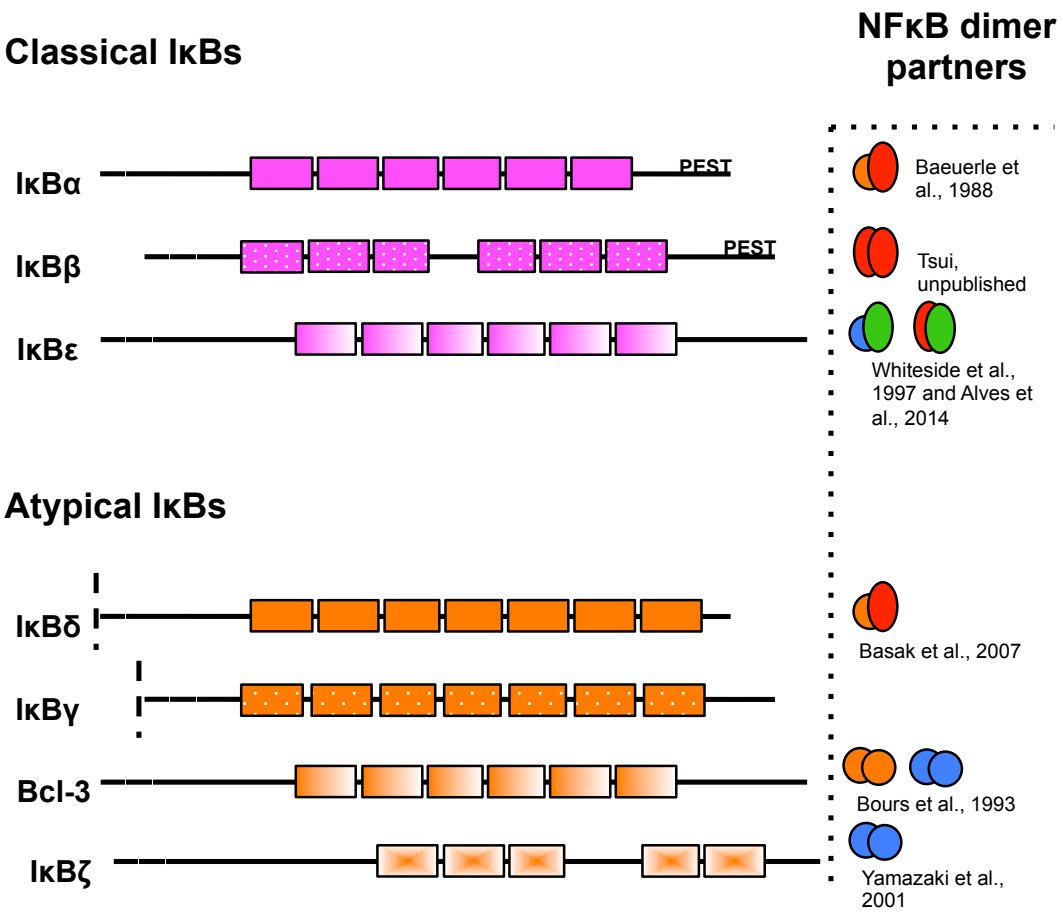


Figure 3.1. The Inhibitors of κB.
Schematic diagram showing different IκB proteins that contain several ankyrin repeats (as indicated by colored boxes). The right panel shows preferential binding partners of the IκBs.

binding partners (Figure 3.1.) and are transcriptionally regulated by different mechanisms (Whiteside and Israel, 1997). For example, the RelA: p50 heterodimer is primarily regulated by IκBα (Karin and Ben-Neriah, 2000) while IκBε controls RelA:RelA and RelA:cRel dimers (Whiteside et al., 1997). The key mechanisms that control IκB degradation and subsequent NFκB activation were revealed nearly 20 years ago. In the case of IκBα, the most well studied IκB, inflammatory stimuli trigger activation of the IκB kinases (IKK), which phosphorylate IκBα serine 32 and 36 within the signal responsive domain (SRD) (Brown et al., 1995; DiDonato et al., 1996). Phosphorylation leads to ubiquitination on lysine 21 and 22 (Scherer et al., 1995) causing 26S proteasomal degradation of IκBα, allowing freed NFκB to accumulate in the nucleus and activate transcription of a large number of genes (Ghosh et al., 1998). However, unbound IκBα is degraded rapidly (Pando and Verma 2000; O'Dea et al., 2007; Mathes et al., 2008). This stimulus-independent turnover of IκBα was shown to be important for NFκB homeostasis (O'Dea et al., 2007) and signal responsiveness (O'Dea et al., 2008, Loriaux et al., 2013). The incompletely folded C-terminus of free IκBα was shown to be necessary for turnover (Mathes et al., 2008, Ferreiro et al., 2007), and mutations towards the ankyrin repeat consensus that increase its foldedness in vitro, prolong IκBα half-life in vivo (Truhlar et al., 2008, Mathes et al., 2010).

In Chapter 2, we reported the identification of a degradation signal located in the C-terminus region of IκBα, that could be transferred to the either N- or C-terminus of the heterologous globular protein GFP. This degron was shown to mediate proteasomal-dependent but ubiquitin-independent degradation, and was located in

ankyrin repeat (AR) 6 encompassing an eleven amino acid sequence of this AR (IκBα 251-262). Furthermore, we showed that the degron of IκBα has a non-redundant function in the degradation pathway of the free full-length protein. However, we did not explore how our degron relates to the folding mutants, (Truhlar et al., 2008, Mathes et al., 2010) which appear to counteract its activity.

To examine the function of the degron in stimulus-responsive NFκB signaling we used an established model of the IκB-NFκB signaling module (Kearns et al., 2006) to generate predictions and pursued a synthetic biology approach to test the model predictions. By fusing the degron to stable IκB family member IκBδ, and reconstituting IκB-deficient cell lines, we found that the degron itself combined with the signal-responsive domain of IκBα does not recapitulate IκBα function. Interestingly, though the degron is required for constitutive degradation of free IκBα (O'Dea et al. 2007; O'Dea et al., 2008; Mathes et al., 2008), its activity must be regulated by NFκB to ensure proper IκBα-mediated control of NFκB activity. Our work delineates the identity of a ubiquitin-independent degron and its mode of regulation through protein-protein-interaction catalyzed protein folding.

RESULTS

Degron makes IκBδ highly unstable even in the presence of NFκB

Since the degron's solvent exposure is obscured when IκBα is bound to NFκB (Huxford et al., 1998; Croy et al., 2004; Truhlar et al., 2006; Ferreiro et al., 2007), and free and bound IκBα are known to have vastly different half-lives in cells (Pando and

Verma, 2000; O'Dea et al., 2007; Mathes et al., 2008), we sought to determine whether regulation of the degron's activity is in fact important for NFκB signaling dynamics. To this end, we pursued a synthetic biology approach based on the IκB family member, IκBδ (Figure 3.2.). Previous work on IκB proteins has shown that IκBδ, which can bind the same NFκB containing dimers (RelA: p50) as IκBα (Figure 3.1), is fully folded in solution and has a long half-life (Basak et al., 2007; Shih et al., 2009). However, IκBδ has previously not been studied in an *nfkb*$^{-/-}$ cell system, therefore validation experiments were needed. *nfkb*$^{-/-}$ cells expressing the ankyrin repeat domain of p100 (480-765), full-length IκBα, as well as SRD-δ-AR6, an IκBδ chimera that had the signal-responsive domain (SRD) of IκBα attached to its N-terminus and the IκBα AR6 degron attached to its C-terminus, were designed and cultured. Interestingly, while IκBδ was stable in both cell lines, the chimera was destabilized in cells not only deficient in NFκB but also – unlike IκBα - containing NFκB (cells deficient in IκBα and IκBε, hereafter referred to as *α/ε*$^{-/-}$; Figure 3.3. panel a). Since SRD-δ-AR6 was difficult to detect *via* immunoblot, we designed an experiment in which the stable IκBδ and the unstable SRD-δ-AR6 were treated with either MG132, CHX or pretreated with MG132 followed by CHX treatment in order to ensure that the chimera was indeed expressed in both cell lines. When these cell lines were treated with MG132, the chimera could be detected (Figure 3.3. panel b), indicating that SRD-δ-AR6 was in fact expressed and was particularly unstable.

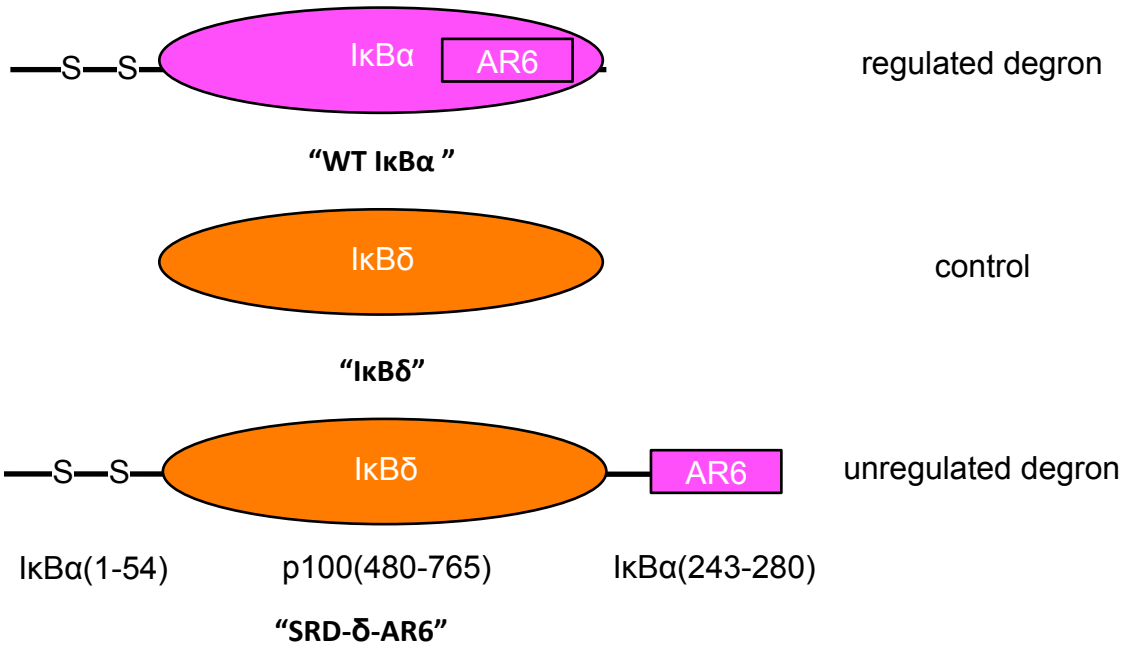


Figure 3.2. Overview of constructs used in Chapter 3.
Three constructs were used in the following experiments. Full-length wild-type IκBα, the ankyrin repeat domain of p100 (480-765) which will be referred to as IκBδ and, the chimera SRD-δ-AR6, which is composed of IκBα (1-54)-p100(480-765)-IκBα(243-280).

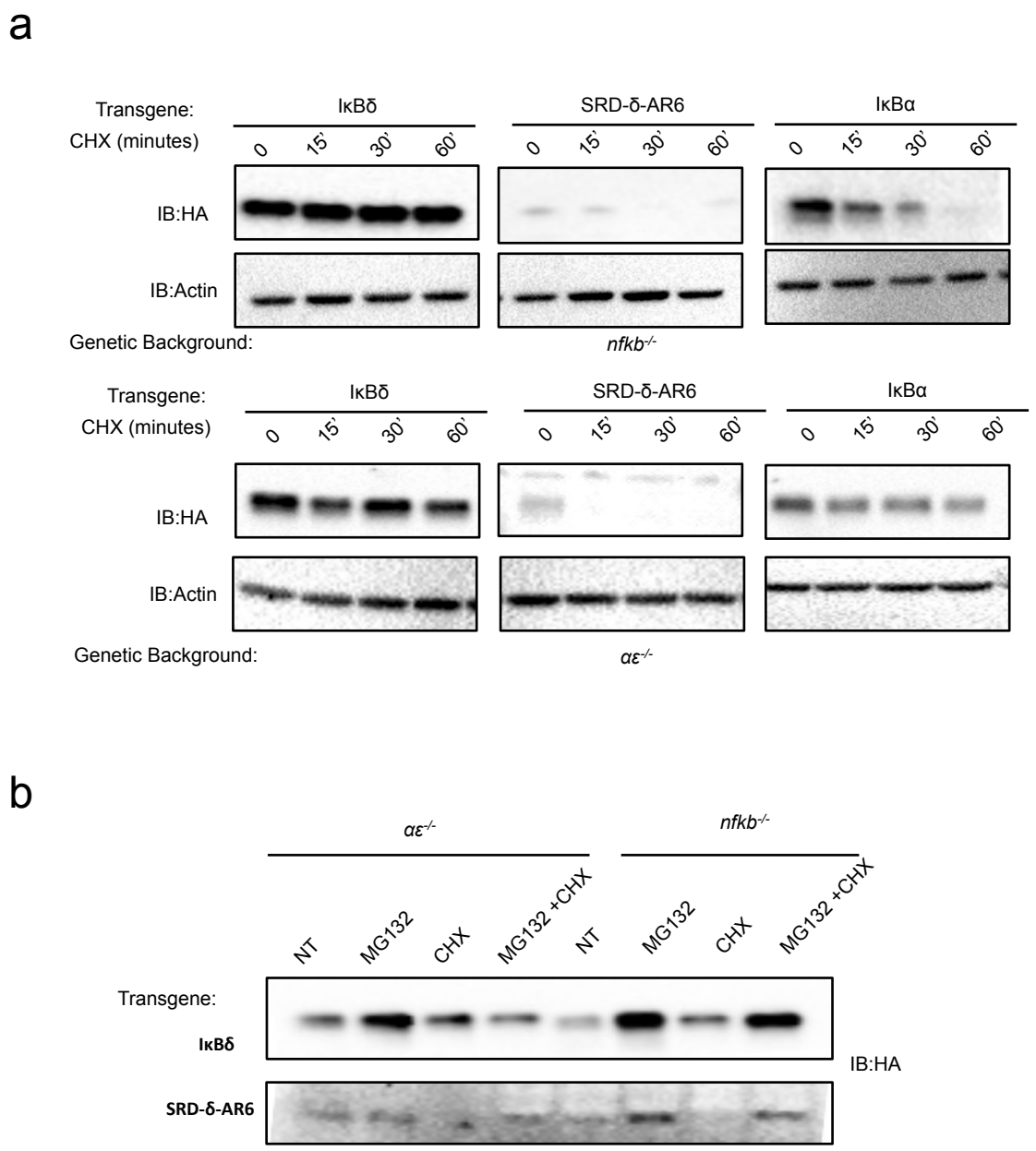


Figure 3.3. The degron makes IκBδ highly unstable even in the presence of NFκB.
(a) Immunoblots for hemagglutinin (HA) tag of whole cell extracts prepared from *nfkb*$^{-/-}$ or *IκBα/IκBε*$^{-/-}$cells expressing IκBδ, SRD-δ-AR6 or IκBα, treated with cycloheximide (CHX). (b) Immunoblots for HA tag of whole cell extracts prepared from *nfkb*$^{-/-}$ or *IκBα/IκBε*$^{-/-}$ cells expressing IκBδ or SRD-δ-AR6: not treated (NT), treated with the proteasome inhibitor MG132, the ribosomal inhibitor CHX or co treated with MG132 and CHX

IκBδ is largely cytoplasmic and cannot activate NFκB without the SRD and AR6 of IκBα

As alterations in ARD of p100 have been shown to effect nuclear shuttling and certain mutations can enhance nuclear accumulation (Liao and Sun, 2003). We set out to determine the cellular localization of the chimera, as well as the ARD of p100 (IκBδ). Cells were stimulated with tumor necrosis factor (TNFα), a cytokine that activates NFκB through the canonical pathway, and examined the cytoplasmic and nuclear fractions of the cells (Figure 3.4. panel a). As expected, TNFα induced the degradation of IκBα in the cytoplasmic fraction. IκBδ protein abundance did not noticeably change in response to the cytokine, but appeared to be largely cytoplasmic; in contrast, SRD-δ-AR6 proved to be difficult to detect in both NFκB containing ($\alpha/\varepsilon^{-/-}$) and NFκB deficient cells ($nfkb^{-/-}$), as expected from the results of our previous experiment (Figure 3.3. panel b). Furthermore, we examined NFκB activity using the nuclear fractions from TNFα stimulated cells *via* electrophoretic mobility shift assay (EMSA) and determined that while IκBα and SRD-δ-AR6 seemed to induce NFκB activity, IκBδ did not (Figure 3.3. panel b).

An unregulated degron impairs IκB's ability to control basal NFκB activity

Using an established kinetic model of the IκB-NFκB signaling module (Hoffmann et al., 2000; Kearns et al., 2006; O'Dea et al., 2007), computational simulations in wild type or IκB mutant conditions were performed for TNF simulation, with NFκB activity as the output, to determine whether free IκB has an

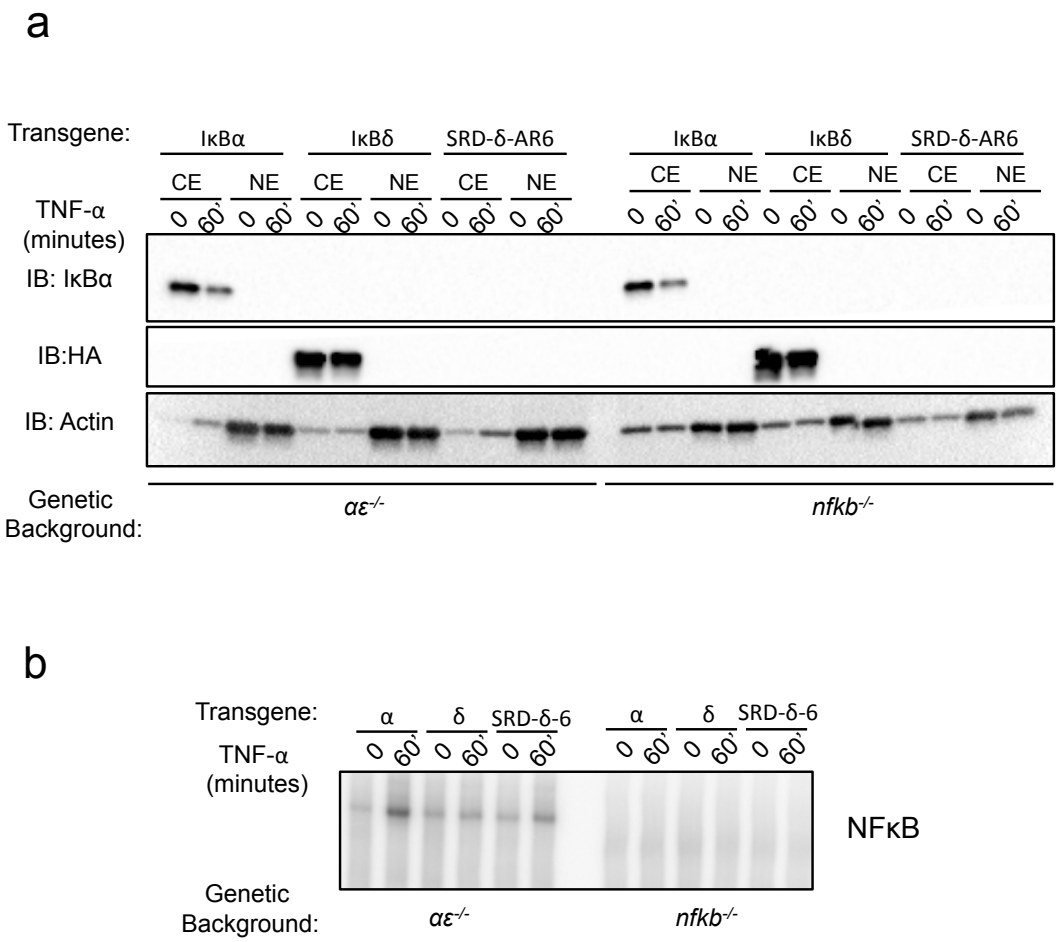


Figure 3.4. IκBδ is largely cytoplasmic and it cannot activate NFκB without the SRD and AR6 of IκBα.

(a) Immunoblot of cytoplasmic and nuclear fractions of *α/ε*$^{-/-}$or *nfkb*$^{-/-}$ cells expressing IκBα, IκBδ (p100(480-765)) or SRD-δ-AR6 (IκBα(1-54)-IκBδ-IκBα (243-280)) treated with 1ng/mL TNFα for indicated times. (b) NFκB activity as measured by EMSA of nuclear extracts from *α/ε*$^{-/-}$cells expressing IκBα, IκBδ, or SRD-δ-AR6 treated with 1ng/mL TNFα. *nfkb*$^{-/-}$ cells are shown as a control.

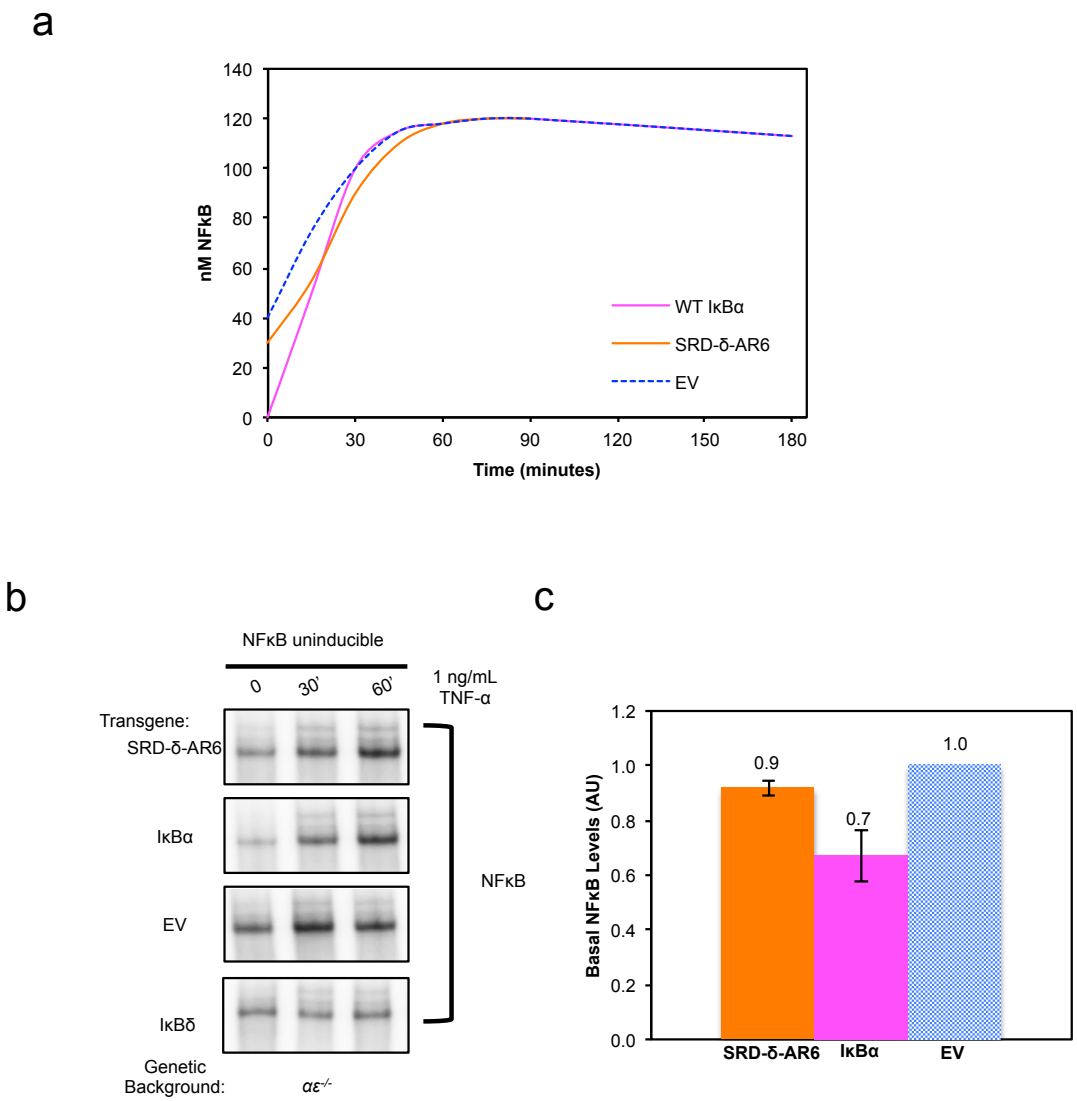


Figure 3.5. An unregulated degron impairs IκB's ability to control basal NFκB activity. (a) Computational simulation of NFκB activation over a 180-minute time course. IκBα is depicted in pink, SRD-δ-AR6 is in orange and empty vector (EV) is shown in blue dotted line. (b) NFκB activity as measured by EMSA of nuclear extracts from $\alpha/\varepsilon^{-/-}$cells expressing IκBα, IκBδ, SRD-δ-AR6, or EV treated with 1ng/mL TNFα for indicated times. (c) Quantification of basal NFκB levels. Error bars indicate standard deviation calculated from 3 separate experiments.

effect on NFκB activation. With long IκB half-lives, little NFκB activation was seen as the resulting IκB overexpression functioned as a transdominant inhibitor; with short IκB half-lives, signaling was recovered, albeit the basal NFκB activity was predicted to be elevated (Figure 3.4. panel a).

Using the IκB variants, we further investigated the effect IκB half-life has on NFκB activation *in vivo* downstream of TNF signaling. As expected, the fully stable IκB functioned as a transdominant inhibitor, suppressing NFκB activation, compared to the IκBα positive control (Figure 3.4. panel b). In contrast, the destabilized chimera did allow for NFκB signaling, but we noted an elevated basal level of NFκB activity (Figure 3.4. panel c), as predicted by the kinetic model (Figure 3.4. panel a). These results support the notion that NFκB-mediated control of IκBα half-life is critical for proper regulation of NFκB activity in basal and stimulus-induced conditions.

NFκB feedback provides further insight on the effect of an unregulated degron

In cells, IκBα is expressed from promoters that are highly NFκB inducible, thus providing negative feedback that may terminate NFκB activity (Kearns et al., 2006). The retroviral transgentic system that we established in $\alpha/\varepsilon^{-/-}$ cells did not allow for NFκB feedback and therefore, the kinetic model could only predict slight differences in basal levels. Therefore, we sought to explore the effect of an unregulated degron on NFκB signaling dynamics that are largely shaped by the NFκB-IκBα negative feedback loop. Kinetic model simulations predicted that while

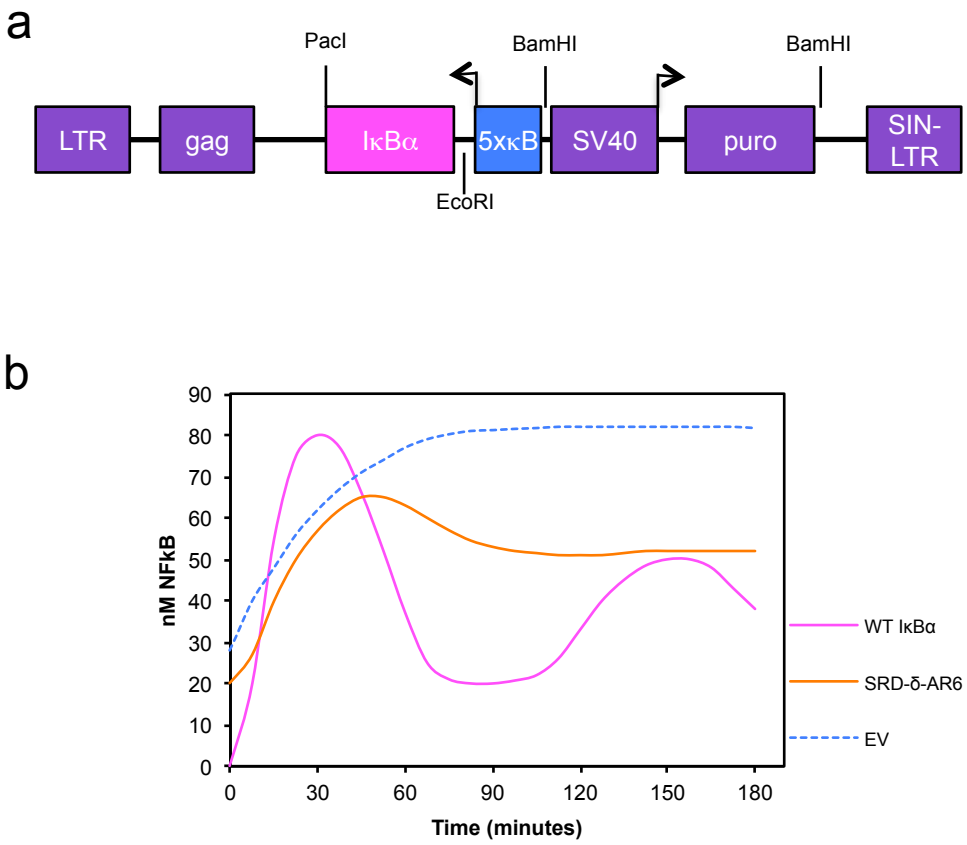
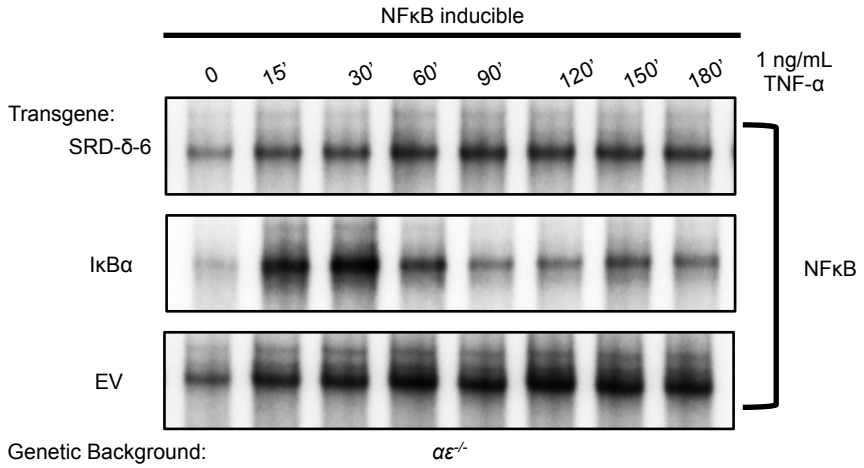


Figure 3.6. Model predicts that even within a NFκB feedback system, the unregulated degron impairs IκBα's function.
(a) Schematic of a murine Moloney virus-based vector that was modified to express the IκB transgene under the control of five κB sites. (b) Computational simulation of NFκB activation (under NFκB feedback) over a 180-minute time course. IκBα is depicted in pink, SRD-δ-AR6 is in orange and empty vector (EV) is shown in blue dotted line.

a



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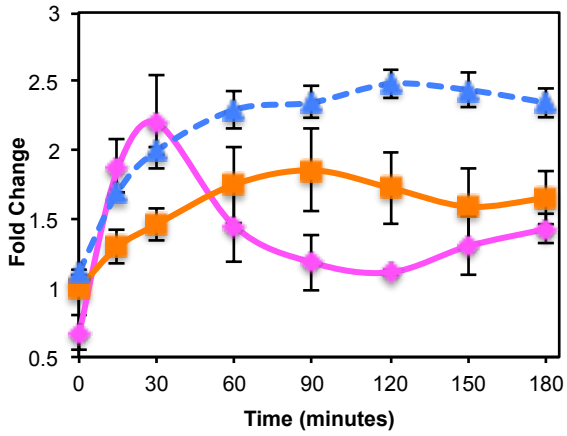


Figure 3.7. *In vivo* data confirms kinetic model's prediction.
(a) NFκB activity as measured by EMSA of nuclear extracts from $\alpha/\varepsilon^{-/-}$cells expressing IκBα, SRD-δ-AR6, or EV in 5xκB vector, treated with 10ng/mL TNFα for indicated times. (b) Quantification of NFκB levels. Error bars indicate standard deviation calculated from 3 separate experiments.

a form of IκB harboring a de-regulated degron may provide some degree of post-induction attenuation, the extent and timing of this attenuation is defective, such that oscillations normally seen with wild type IκBα cannot be sustained (Figure 3.6. panel b). We tested these predictions *in vivo* using a retroviral construct system that expresses the IκB variants under the control of five κB sites (Figure 3.6. panel b). Indeed, even with NFκB feedback, the unregulated degron impaired IκB's function (Figure 3.7. panel a). The experiment provides data that is consistent with the kinetic model. (Figure 3.7. panel b). These data suggest that that NFκB-mediated control of IκB half-life is essential for characteristic NFκB signaling dynamics.

DISCUSSION

The degron of IκBα must be regulated to provide efficient regulation of NFκB

In Chapter Two, we revealed the molecular determinants of a ubiquitin-independent degron. Here, we have shown that this regulation is necessary for the proper control signaling and that de-regulating the degron leads to faulty timing and higher basal levels of NFκB. Previous reports have shown that high degradation rates of free IκBα warrant low levels of excess IκBα (O'Dea et al., 2007), which helps tune the cellular responsiveness of NFκB (O'Dea et al., 2008). We have demonstrated in our study that the degron is the signal responsible for high flux, and unless its activity is regulated, the fluxing IκB does not provide for efficient inhibition of NFκB. By regulating the degrons activity via folding that is triggered by the interaction with NFκB (Huxford et al., 1998; Croy et al., 2004; Truhlar et al., 2006; Ferreiro et al., 2007), IκBα is an effective inhibitor of NFκB in a basal state and for post-induction

repression, while still allowing for IKK-responsiveness.

MATERIALS AND METHODS

DNA constructs.

IκBα constructs were cloned into the retrovirus vector pBabe-puro between the restriction sites EcoRI and SalI. IκBδ was cloned between the restriction sites EcoR. For signaling studies, a murine Moloney virus-based vector was modified to express the IκB transgene under the control of five κB sites.

Cell Culture.

Immortalized 3T3 cells were cultured as described in Chapter 5.

Biochemical Assays and Reagents.

Cells treated with cycloheximide (Calbiochem) were used at 10 μg/mL in 100%EtOH. For signaling studies, 1 or 10 ng/mL recombinant murine TNFα (Roche) was used. After treatment with cycloheximide, cells were lysed in RIPA buffer and equivalent protein amount were subjected to immunoblot analysis using Clarity Western ECL substrate (BioRad). IκBα was probed with sc-371 (Santa Cruz Biotechnology), while IκBδ and SRD-δ-AR6 were probed using HA 16b12 (Covance). Nuclear and cytoplasmic extracts were prepared as described in Chapter 6 and used for immunoblot analysis or electrophoretic mobility shift assays (EMSA) as described in Chapter 5. EMSA signals were quantitated using ImageQuant software (GE Healthcare)

Computational modeling.

The web interface mathematical model of the IκB-NFκB signaling module (http://signalingsystems.ucsd.edu/models-and-code/) was used (version 1.2; Kearns et al., 2006) to generate Figure 3.5 and 3.6. Total IKK Time (simulation phase) was

changed to 40 μM and mRNA half-life and synthesis rates were halved to simulate IκBα, SRD-δ-AR6, and empty vector. To simulate SRD-δ-AR6 we set the NFκB bound degradation rate equal to the unbound degradation rate and to simulate EV we changed the initial concentration of nuclear NFκB to 85nM.

ACKNOWLEDGEMENTS

Riku Fagerlund created the 5xκB vector that was used for signaling studies. I performed the computational simulations with guidance from Alexander Hoffmann. Most content from this chapter has been submitted for publication with the work from Chapter 2 in which R. Lewis will have authorship and Alexander Hoffmann will be the corresponding author.

Chapter 4:

Describing the Half-Life Control of the Other Canonical IκBs in Their Free Form: The Tale of IκBβ and IκBε

ABSTRACT

The IκB proteins regulate NFκB activity. Specifically, IκBα, -β, or –ε are responsible for the control of NFκB by associating with NFκB dimers. Whereas both basal and stimulus induced degradation of IκBα has been studied in detail, little is known about the mechanisms governing IκBβ and IκBε. Here we examine the basal turnover of IκBβ and IκBε using a retroviral transgenic system. We stably expressed mouse IκBβ and IκBε in mouse embryonic fibroblasts deficient in the canonical NFκB proteins: p50, cRel, and RelA. We found that when we introduced these wild-type proteins into *nfkb*$^{-/-}$ cells, IκBβ exhibits a short half-life similar to free IκBα, and, strikingly, IκBε is stable. In addition, unlike free IκBα, no stable truncation of IκBβ could be made indicating that IκBβ does not have a degron, or that its degron is more complex than that of IκBα. Furthermore, in the case of IκBε, when the proteasome activator PA28γ was overexpressed, destabilization of IκBε was observed suggesting a specialized function of IκBε in cells expressing the immunoproteasome.

INTRODUCTION

The activation of pro-inflammatory gene expression by NFκB is regulated by the association of NFκB with its inhibitor proteins: IκBα, IκBβ, or IκBε. These IκBs are deemed the "classical" or canonical IκBs and are responsible for controlling the activity of NFκB in response to inflammatory signals. Their synthesis and degradation rates are carefully regulated in order to maintain proper NFκB activity. IκBα and IκBβ were discovered using a biochemical activity test whereby chromatic fractions were tested for their ability to inhibit the DNA binding activity of NFκB. The fractions that showed an effect on NFκB were then purified via chromatography and single polypeptides were isolated (Baeuerle and Baltimore, 1988; Thompson et al., 1995; Zabel and Baeuerle, 1990). IκBε was discovered sometime later using sequence homology and was characterized using similar biochemical analysis (Whiteside et al., 1997).

IκBα, IκBβ and IκBε proteins all contain six copies of a motif, the ankyrin repeat, which interacts with the RHD of the rel/NFκB proteins. Ankyrin repeats are typically a 33 amino acid consensus sequence consisting of two alpha helices separated by loops that appear in a large number of proteins. The amino-terminal regions of the IκBs contain a signal response domain (SRD) that is mostly conserved in the classical IκBs. In the SRD there are two serine residues and two lysine residues that are critical for IκB responsiveness. The serine residues are the sites of phosphorylation by the IκB kinase, IKK, and the lysine residues are required for ubiquitin conjugation. Similar to IκBα, IκBβ contains a proline (P), glutamic acid (E), serine (S), and threonine (T) rich region at its C-terminus, which has been deemed the

PEST region. Previous studies have associated this region with rapid turnover in cells (Rogers et al., 1986). However, as we saw in Chapter Two, the precise role of this region remains inconclusive.

The stimulus-induced degradation of IκBα has been well studied. Serine residues 32 and 36 are phosphorylated (Brown et al., 1995), which causes the ubiquitination of lysine 21 and 22 by the E3 ligase β-TRCP, triggering proteasomal degradation by the 26S proteasome (Scherer et al., 1995). Similar stimulus induced degradation occurs for IκBβ and IκBε whereby serine 19 and 23 (DiDonato et al., 1996) or serine 18 and 22 (Whiteside et al., 1997) are respectively phosphorylated (Figure 4.1.).

Interestingly, whereas IκBα is rapidly synthesized upon NFκB activation, NFκB-induced transcription of IκBε is delayed (Kearns et al., 2006). As a result, IκBε plays a role in dampening IκBα-mediated oscillations during persistent NFκB activity. Additionally, IκBβ has shown to not have a negative feedback effect on NFκB, but instead has been shown to act as a chaperone in the formation of RelA homodimers (Tsui, unpublished). The IκBα and IκBβ crystal structures show various similarities between the two proteins (Huxford et al., 1998; Jacobs et al., 1998; Malek et al., 2003) and although there is no crystal structure of IκBε, it is assumed (based on likenesses of sequence) to adopt an analogous structure. Given the lack of data on free IκBβ and IκBε, we used the previously established *nfkb*$^{-/-}$ cell system to investigate the half-life control of free IκBβ and IκBε half-life. Here, using biochemical analysis, we show that while IκBβ may have degradation kinetics akin to IκBα, free IκBε is in fact a

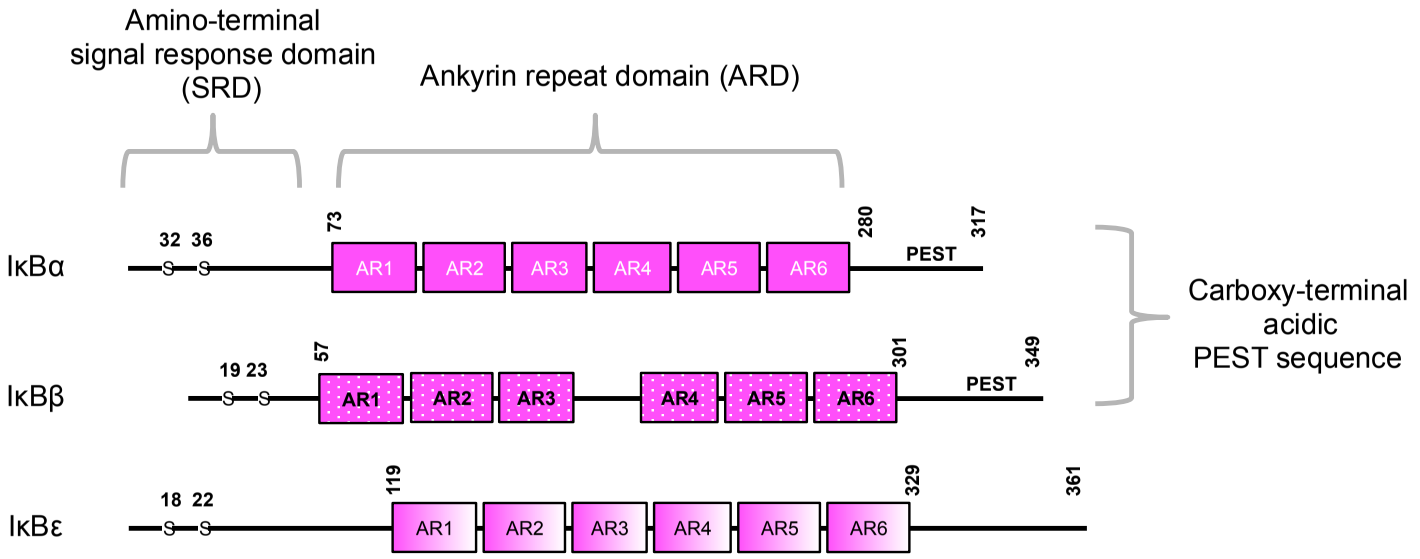


Figure 4.1. The classical inhibitors of NFκB.
Domain organization in the IκB family of inhibitor proteins. The positions of IKK phosphorylation sites are highlighted as well as the approximate location of domain boundaries in human proteins.

stable protein. These data suggest that IκBε is less suited to ensure NFκB inhibition in resting cells.

RESULTS

In MEFs unbound IκBβ shows parallel degradation kinetics to IκBα

To characterize the degradation mechanism of unbound IκBβ, we used a retroviral transgenic system to introduce murine IκBβ into immortalized mouse embryonic fibroblasts deficient in the NFκB proteins known to associate with the canonical IκBs. This *nfkb1*$^{-/-}$*rela*$^{-/-}$*crel*$^{-/-}$ cell line will be referred to hereafter as *nfkb*$^{-/-}$. By treating the cells with cycloheximide (CHX), an inhibitor of translation, the approximate half-life of free IκBβ was determined. This revealed that when wild-type IκBβ is expressed in theses cells, it is rapidly degraded. As decay can occur in either a linear or in an exponential fashion, we quantified our data and used statistical analysis to determine which type of degradation IκBβ undergoes. Figure 4.2. panel "b" illustrates that the coefficient of determination (R^2) is closer to 1 in the exponential decay scenario, indicating that IκBβ decreases at a rate proportional to its current value. One characteristic of exponential decay is protein half-life, which is the time required for the decaying quantity to fall to one half its initial value. This can be written mathematically as $t_{1/2} = \frac{\ln 2}{\lambda}$ where λ is the exponential decay constant extracted from the equation $N\ (t) = N_0 e^{-\lambda t}$ which was fit in Microsoft Excel (Figure 4.2.).

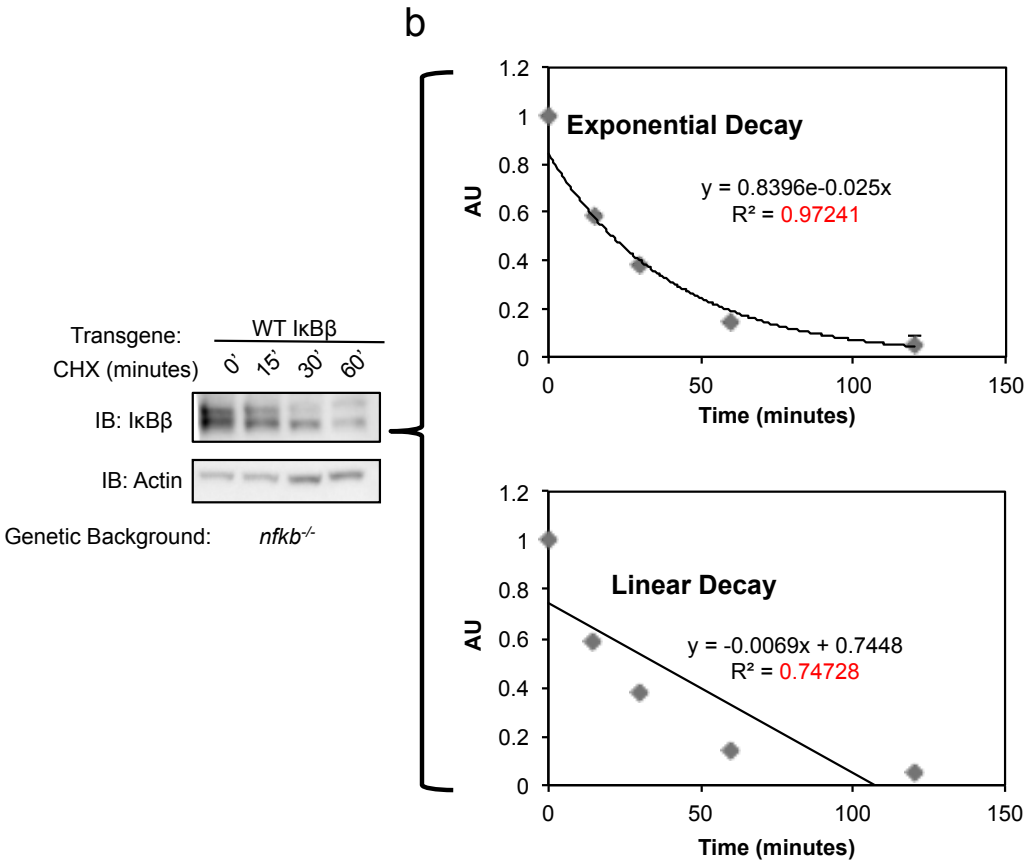


Figure 4.2. Unbound IκBβ experiences exponential decay.
(a) Immunoblots for IκBβ of extracts prepared from *nfkb*$^{-/-}$ cells expressing wild-type murine IκBβ treated with CHX. * Indicates nonspecific bands associated with this antibody. (b) Quantitation 3 different experiments, error bars indicate standard deviation. Top panel: exponential regression fit, R-squared value indicated in red. Bottom panel: linear regression fit, R-squared value indicated in red.

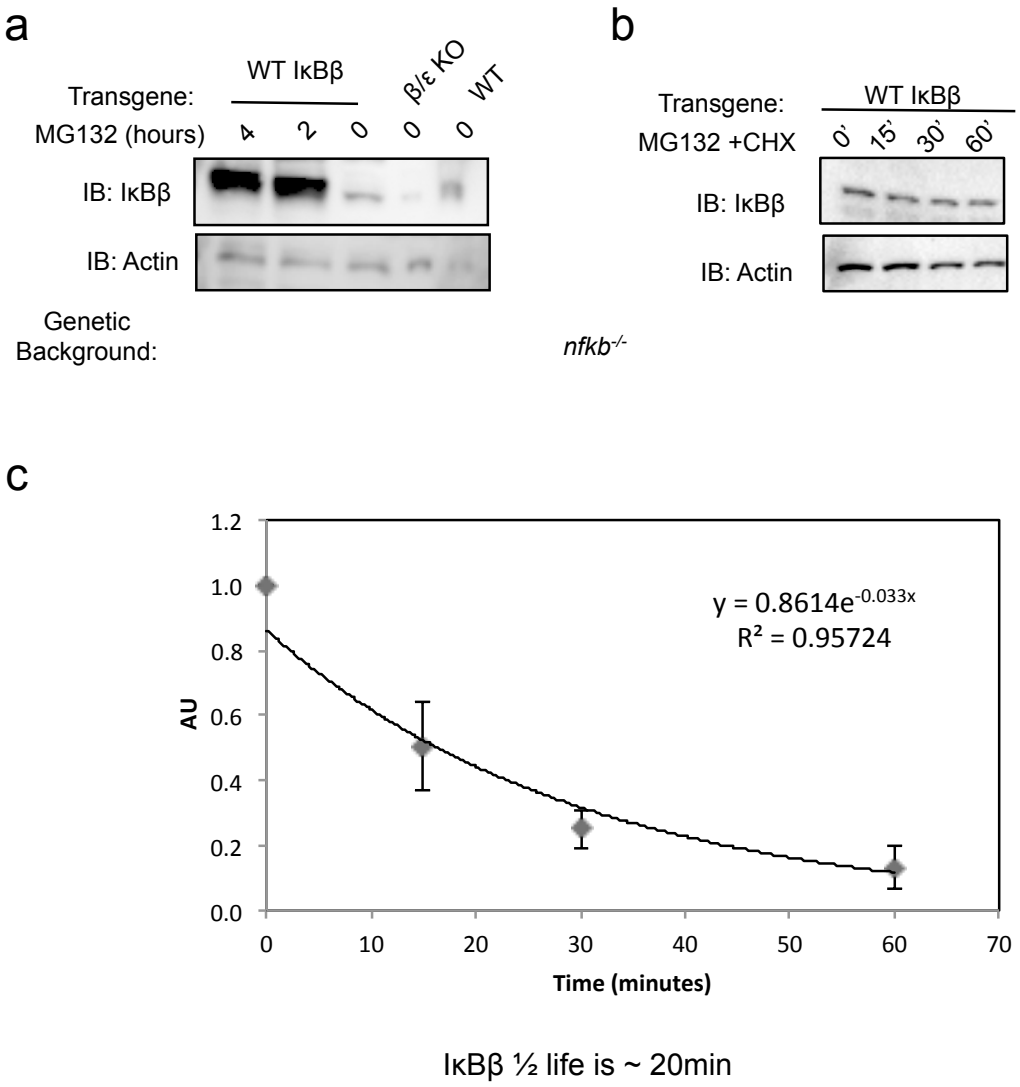


Figure 4.3. Free IκBβ is degraded in a proteasome-dependent manner.
(a) Immunoblots for IκBβ of extracts prepared from *nfkb*$^{-/-}$ cells expressing wild-type murine IκBβ treated with MG132 for the indicated times. β/ε knockout cells as well at WT cells shown as a control. (b) Immunoblot for IκBβ of protein extracts from *nfkb*$^{-/-}$ cells expressing IκBβ pretreated with MG132 for an hour then treated with CHX for the indicated times. (c) Quantification of 3 CHX experiments used to calculate half-life, error bars indicate standard deviation. Approximate half-life of IκBβ is indicated.

Since free IκBα is degraded in a proteasome dependent manner (Alvarez-Castelao and Castaño, 2005; Mathes et al., 2008; O'Dea et al., 2008; Figure 2.1. and Figure 2.2.), we next sought to determine whether this was the case for unbound IκBβ. Treatment of *nfkb*$^{-/-}$cells with the proteasome inhibitor MG132 led to the accumulation of free IκBβ (Figure 4.3. panel a), and prevented its degradation when cells were co-treated with CHX (Figure 4.3. panel b). Based on our biochemical data, we calculated the half-life of IκBβ to be 15-20 minutes (Figure 4.3. panel b).

Unlike IκBα, truncations of IκBβ do not render it more stable

IκBα and IκBβ show similar domain architecture (Malek et al, 2003). Both proteins contain a centrally located ankyrin repeat domain (ARD) consisting of six ankyrin repeats. The ARD is flanked by a signal response domain (SRD) on its amino-terminus and on its carboxy-terminus it contains an acidic region rich in proline, glutamic acid, serine, and threonine residues. However, IκBβ contains a nonconserved 47 amino acid linker region between ankyrin repeat 3 and 4. Despite this, the crystal structure of IκBβ/RelA homodimer complex shows many similarities with that of the previously determined IκBα/RelA:p50 heterodimer complex (Malek et al., 2003; Huxford et al., 1998; Figure 4.4. panels a and b). As IκBα and IκBβ show structural similarities, we used our knowledge of the IκBα degron to determine whether a degron was present in IκBβ rendering it unstable when not in complex with NFκB.

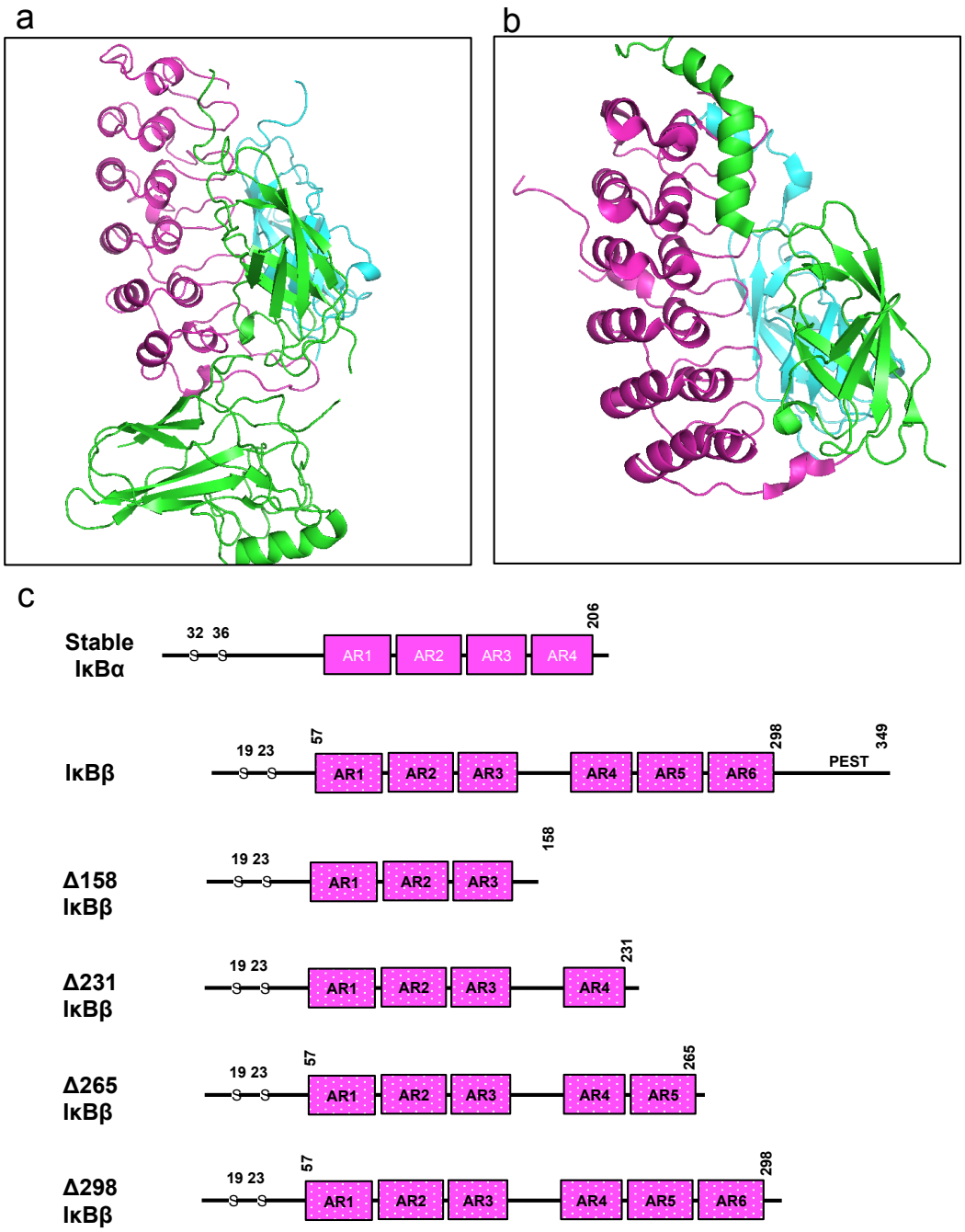


Figure 4.4. The crystal structure of IκBα and IκBβ give insight into IκBβ truncations. (a) A ribbon diagram representation of the crystal structure of IκBα (pink) bound to NFκB (p50, cyan; p65, green) (b) A ribbon diagram representation IκBβ (pink) bound to NFκB (RelA, cyan; RelA, green) not shown is disordered linker region between AR3 and 4. (c) Domain organization of the various truncations of IκBβ used in this study. *AR*, ankyrin repeat.

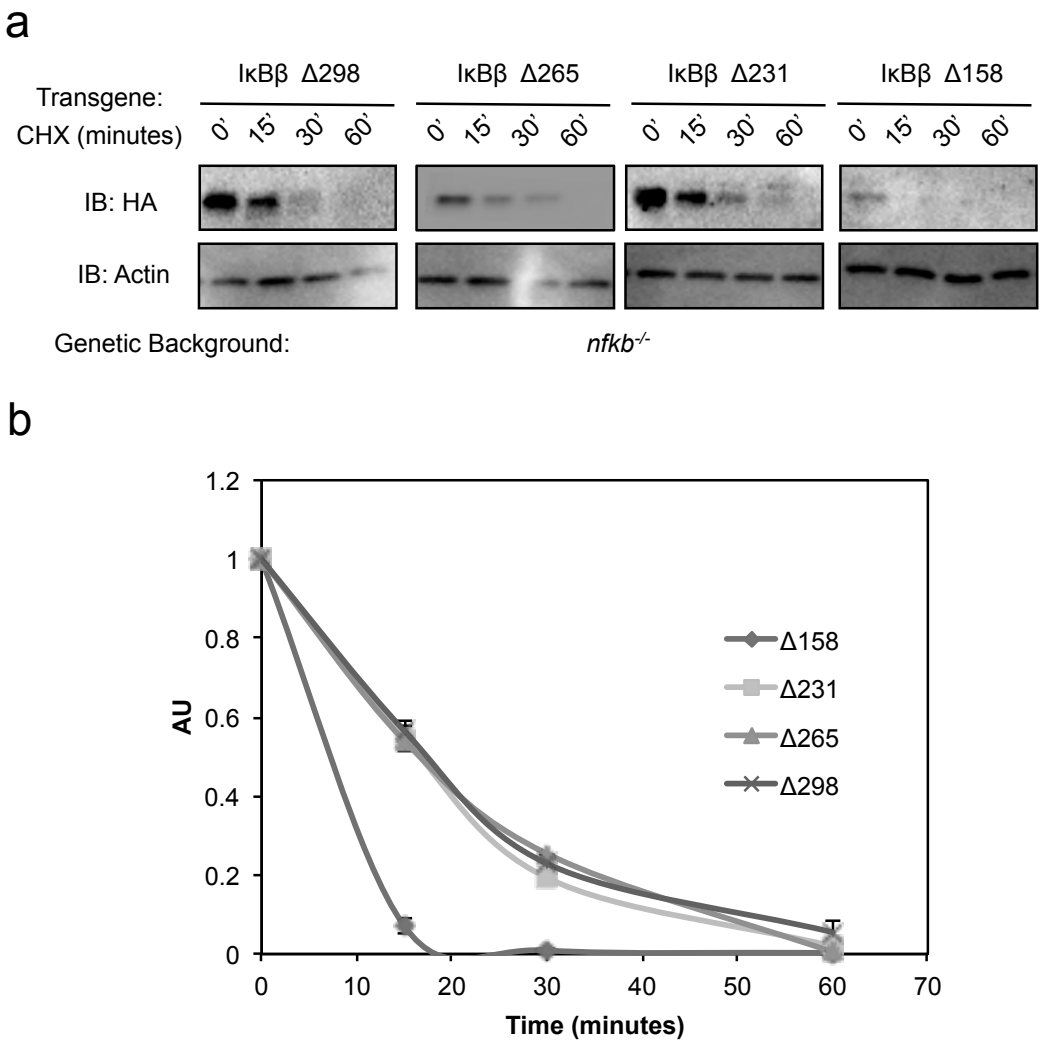


Figure 4.5. Truncations in IκBβ do not render it more stable.
(a) Immunoblots for hemagglutinin (HA) tag of extracts prepared from *nfkb*$^{-/-}$ cells expressing IκBβ Δ298, IκBβ Δ265, IκBβ Δ231, or IκBβ Δ158 treated with CHX. (b) Quantification of 3 experiments, error bars indicate standard deviation.

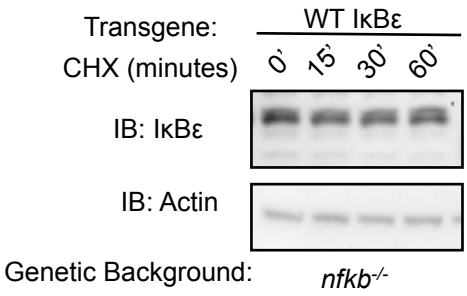
Previous work on free IκBα revealed that when the 5th and 6th ankyrin repeat domains of IκBα were removed entirely, unbound IκBα is dramatically stabilized (Mathes et al., 2010; Figure 2.3.). To test whether similar truncations could stabilize unbound IκBβ, we created four constructs, Δ158, Δ231, Δ265, and Δ298 (Figure 4.4. panel c). Δ158 removes the entire 4th, 5th, and 6th repeats of IκBβ as well as half of the unfolded linker region. Δ231 was truncated after AR 4; Δ265 was truncated after AR 5 and Δ298 removed the PEST region of IκBβ but kept the ARD intact. Surprisingly, unlike unbound IκBα, no stable truncation of IκBβ could be made (Figure 4.5.).

Free IκBε degradation rate is much slower when compared to IκBα or IκBβ

Next, we explored the degradation mechanism of unbound IκBε. Using NFκB deficient cells we expressed murine full-length wild-type IκBε. Unexpectedly, following a 60-minute CHX time course, it was determined that free IκBε was in fact stable (Figure 4.6.). After experimental replication, we came to the conclusion that free IκBε must have a different mechanism of degradation than unbound IκBα and IκBβ.

In immune cells, knockouts of the proteasome activator PA28γ show elevated levels of the NFκB inhibitor IκBε (He et al., 2012; Yu et al., 2010). PA28γ knockouts also show immunological defects as brought out by experimental autoimmune encephalomyelitis (EAE) (Preckel et al. 1999; Murata et al. 2001; Yamano et al. 2008). This suggests that PA28γ might have an effect on the degradation mechanism of free IκBε. To investigate this hypothesis further we used *nfkb*$^{-/-}$ cells and stably transduced both PA28γ, and IκBε, then treated the cells with either the proteasome

a



b

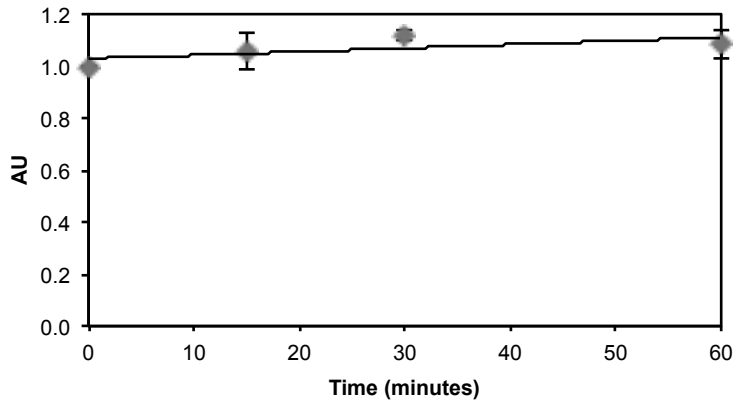


Figure 4.6. Free IκBε is stable in a 60-minute time course.
(a) Immunoblots for IκBε of extracts prepared from *nfkb*$^{-/-}$ cells expressing wild-type murine IκBε treated with CHX. (b) Quantification of 3 CHX experiments used to calculate half-life, error bars indicate standard deviation.

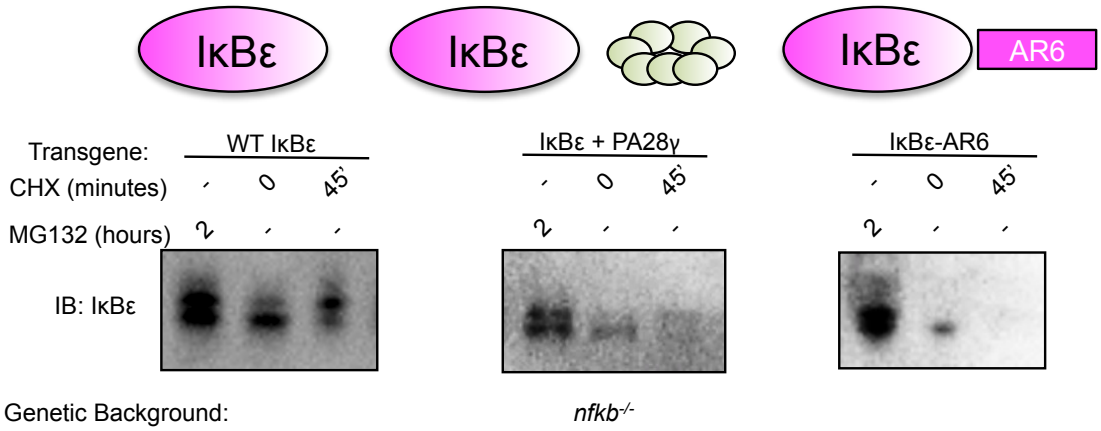


Figure 4.7 Free IκBε can be destabilized in the presence of the proteasome activator PA28γ or when expressing the degron of IκBα (AR6).
Immunoblots for IκBε of extracts prepared from *nfkb*$^{-/-}$ cells expressing wild-type murine IκBε Left panel: Cells treated with MG132 or CHX for the indicated times. Middle panel: PA28γ is coexpressed with WT-IκBε then treated with MG132 or CHX for the indicated times. Right panel: The degron of IκBα (AR6) is linked to the C-terminus of WT-IκBε then treated with MG132 or CHX for the indicated times.

inhibitor MG132 or the inhibitor of translation CHX. Interestingly, when PA28γ was overexpressed we observed a destabilization of free IκBε (Figure 4.6.). In addition to this, when we tagged IκBε with the degron containing ankyrin repeat of IκBα (AR 6; Figure 2.5. panel a), we observed disruption of IκBε stability (Figure 4.6.). These results are consistent with the findings in Chapter 2 that showed the high turnover of the degron could be transferred to a heterologous protein.

DISCUSSION

The inhibitor of NFκB (IκB) family of proteins is known to regulate NFκB activity by cytoplasmic sequestration. The "classical" IκB proteins, IκBα, -β, and –ε are inducibly degraded by inflammatory signals which engage the TNF receptor and toll-like receptor family members to activate canonical, NEMO-dependent IKK activity (Baeuerle and Baltimore, 1996). IκBα is the most responsive of the "classical" IκB proteins followed by IκBε and then IκBβ, which has about half to a quarter of IκBα's responsiveness (Hoffmann et al., 2002). Detailed understanding of stimulus-responsive NFκB activation has been reported; however, the steady state regulation of NFκB by IκBs has yet to be fully understood.

IκBβ degradation is rapid, regulation remains to be discovered

Here we show that unbound IκBβ has similar degradation kinetics to IκBα, and has a half-life of 15-20 minutes. Given that IκBα and IκBβ have structural similarities, we used our knowledge of IκBα's degron (Chapter 2) to investigate the possibility that IκBβ also contains a degron. Our results showed that no truncated

form of IκBβ could be engineered to gain stability. Although this is an interesting result, it can be explained by one of two possible scenarios. T he first scenario is justified by the fact that while IκBα is in its free form, the first four ARs are folded compactly whereas AR 5 and 6 are highly flexible (Ferrerio et al., 2007; Truhlar et al., 2006; Croy et al., 2004). However, no NMR spectroscopy has been performed on free IκBβ due to the fact that it aggregates *in vitro* and therefore the whole IκBβ protein might in fact be incompletely folded when not bound to NFκB. The hypothesis that misfolded proteins are degraded by the proteasome is not novel (Ding and Yin, 2008; Verma and Deshaies, 2000;Benaroudj et al., 2001;Baugh et al., 2009; Asher et al., 2006). However, as we showed in Chapter 2, there has been an increase in the number of proteins that have been found to contain an intrinsic degradation signal, or a "degron" (Ravid and Hochstrasser 2008; Rao et al., 2012; Singh Gautam et al., 2012). This leads us to our second explanation; it is possible that every ankyrin repeat in IκBβ contains a degron. While both rationalizations of IκBβ are thought provoking, no irrefutable conclusions can be made without further examination.

IκBε is stable but can be destabilized in the presence of IκBα's degron or with the over expression of PA28γ

When investigating the half-life control of IκBε, we were surprised to find that it was in fact, stable. However, the overexpression of the proteasome activator PA28γ in *nfkb*$^{-/-}$ cells along with IκBε allowed for degradation of IκBε protein levels. Intriguingly, the cytokine Interferon-γ (IFNγ) transcriptionally induces the proteasome activators PA28α and PA28β, while PA28γ appears to be rapidly proteolyzed

following IFNγ stimulation (Tanahashi et al., 1997). It would be of interest to determine the effect that IFNγ treatment has on the *nfkb*$^{-/-}$+ IκBε + PA28γ cells, in order to further comprehend this pathway. Since PA28γ knockout mice have immunological defects (He et al., 2012; Yu et al., 2010), a subsequent question arises what happens if PA28γ knock out mice are crossed with IκBε knock out mice? It is possible that the combined loss of PA28γ and IκBε would rescue the defects seen in the PA28γ knockout. In addition, we could broaden our understanding of the function of this pathway by using an immunological model. IκBε reduces B-cell proliferation, therefore one could co-stimulate with IFNγ (and LPS) and measure IκBε accumulation and B-cell proliferation. If enhanced proliferation was correlated with a loss of IκBε we could further probe the pathway by using an IκBε knockout B-cell system.

MATERIALS AND METHODS

DNA constructs.

IκBβ and IκBε were PCR amplified from mouse cDNA using the primers 5'- CCGCGGATCCGCCACCATGGCCGGGGTCGCGTGCTTGGG-3' and 5'-CCCGGAATTCTCAGGCAGGGTTGGGGTCATCAGG -3'and 5'-CCACGCGTCGACGCCACCATGTCGGATGCGCGGAAGGGGCCGGACG-3' and 5'- CCACGCGTCGACTCAGTCAGTACATAGCAGTGGTTTGCC-3' for IκBε. Amplified IκBβ and IκBε were then cloned into pBABEpuro, using BamHI and EcoRI and SalI and SalI, respectively.

Cell Culture.

Immortalized 3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum, 1% L-glutamine, and 1% penicillin and streptomycin at 5% CO_2, 37^{o}C. Cells treated with cycloheximide (Calbiochem) were used at 10 μg/mL in 100% EtOH. For proteasome inhibition, 10μM MG132 (Sigma) was used. For virus production, Plat-e cells (Morita et al., 2002) were transiently transfected with PEI (Reed et al., 2006) with 10 μg of pBABE and allowed to grow for 48 hours. Filtered virus was placed onto target cells along with 4μg/mL Polybrene (Sigma). Infected cells were selected with 2.5 μg/mL puromycin (Sigma).

Biochemical Assays.

After treatment with cycloheximide, cells were lysed in RIPA buffer. Cell extract was separated on a 4-15% gradient SDS-PAGE and transferred to PVDF membrane. IκBβ was probed with sc-945 (Santa Cruz Biotechnology) and truncations of IκBβ were probed with HA 16b12 (Covance) then followed by HRP conjugate

(Santa Cruz Biotechnology). IκBε was probed with sc-7155 or with 9249S (Cell Signaling). Quantification of immunoblots was performed with ImageJ. Dilution series with knockout extracts assured that Western blot signals were in the linear range.

ACKNOWLEDGEMENTS

PA28γ DNA was a generous gift from Yidan Li. I thank Bing Xia for help in generating IκBβ and IκBε and Bärbel Schröfelbauer for insightful discussion. This work contains data that is in preparation for publication.

Chapter 5:

Conclusions and Future Directions

Cells are capable of rapidly responding to changes in their environment, including the presence of pathogens or noxious conditions. Molecular signaling pathways that regulate these responses show highly dynamic patterns of activity. Indeed, dynamic control of protein degradation rate and protein half-life is a dynamic hallmark of many signaling pathways. In the case of the NFκB pathway, the key regulator of inflammatory responses, the inhibitor proteins IκBα, -β, and -ε are known to be regulated by signal-responsive mechanisms freeing NFκB. Recent work indicates that IκBα is synthesized in excess in resting cells to ensure that NFκB activity remains effectively inhibited (Hoffmann and Baltimore, 2006; Vallabhapurapu and Karin, 2009). Rapid degradation of the excess IκB is critical so that NFκB activation can proceed when inflammatory responses are needed (O'Dea et al., 2007; Mathes et al., 2008; O'Dea et al., 2007).

Cellular protein degradation is catalyzed by the proteasome, a large molecular machine. Most proteins are targeted for degradation by a specific post-translational modification, the covalent attachment of the small molecule ubiquitin (Ciechanover, 2005). However, there is an increasing amount of work that has demonstrated that proteins can undergo degradation without prior linkage of ubiquitin molecules (Orlowski and Wilk, 2003; Jariel-Encontre, 2008). Abnormalities the degradation underlie the pathogenesis of numerous human diseases, therefore the proteasome has been an important target for drug discovery (Ostrowska, 2007; Crawford et al., 2011). However, many inhibitors of the proteasome have limited therapeutic worth due to their high levels of cytotoxicity against normal cells and their lack of selectivity (Chen et al., 2011; Rajkumar et al., 2005). Therefore, the understanding of different

degradation signals that the proteasome utilizes is essential for the evolution of therapeutics as targeted proteasome inhibitors might ease side effects.

In this dissertation, I have investigated the mechanism of degradation that free IκBα, the quintessential inhibitor of NFκB, undergoes. I used these findings to discover the functional relevance of the degradation mechanism and furthermore delved into the method by which other IκBs, namely IκBβ and IκBε, are regulated in their free forms.

In Chapter 2 we identify the degradation signal of IκBα, by employing fundamental biochemical techniques. We identified and characterized the ubiquitin-independent, proteasome-dependent degradation signal of IκBα. We showed that the degron is located in the 6th ankyrin repeat of IκBα and that it is sufficient to cause degradation of a heterologous protein, GFP. Furthermore, we showed that the degron is unique and does not appear to rely on the incompletely folded region of IκBα, but instead relies on specific amino acid residues within ankyrin repeat 6 that are hidden when properly folded. Future work will look to explore the mechanism by which the proteasome recognizes the degron, and additionally how the degradation takes place.

In Chapter 3 we investigated the effect that an unregulated degron had on NFκB activity. Using a combined system of biochemistry, systems biology, and synthetic biology we showed that regulation of the degron is necessary for the proper control signaling and that de-regulating the degron leads to faulty timing and higher basal levels of NFκB. Previous reports have shown that high degradation rates of free IκBα warrant low levels of excess IκBα (O'Dea et al., 2007), which helps tune the cellular responsiveness of NFκB (O'Dea et al., 2008). We demonstrated in our study

that the degron is the signal responsible for high flux, and unless its activity is regulated, the fluxing IκB does not provide for efficient inhibition of NFκB. By regulating the degron's activity via folding that is triggered by the interaction with NFκB (Huxford et al., 1998; Croy et al., 2004; Truhlar et al., 2006; Ferreiro et al., 2007), IκBα is an effective inhibitor of NFκB in a basal state and for post-induction repression, while still allowing for IKK-responsiveness.

In Chapter 4 we investigated whether other canonical IκB proteins in their free form, namely IκBβ and IκBε, underwent similar half-life control as IκBα. We discovered that while IκBβ may undergo similar control as IκBα, IκBε is in fact a stable protein. These results indicated that IκBε maybe less suited to ensure NFκB inhibition in resting cells. Interesting, we found that the overexpression of the immunoproteasome component, PA28γ, destabilized free IκBε. More biochemical and biological work is currently needed to understand the mechanistic detail by which PA28γ mediates free IκBε degradation. These findings provide insight on the mechanisms governing IκBβ and IκBε as well as provide kindling for future projects to come.

Together, these data indicate that every IκB seems to have different degradation control. IκBα contains a degron for 20S mediated proteasomal degradation, which can function regardless of ubiquitin conjugation. It seems as if IκBε contains a degron that targets it to the immunoproteasome, however more work needs to be done to understand the biological implications. Additionally, while IκBβ is unstable, it is unclear what degron, if any, it contains.

This dissertation emphasizes the importance in comprehending the steady-state mechanisms by which proteins are regulated in order to understand how a cell will behave in response to environmental signals. Pharmacologic inhibitors of the proteasome have shown to have an imperative effect on malignant cancers, however many lack selectivity. The work presented here could be important in designing new therapeutics with greater selective activity.

Chapter 6:

Materials and Methods

The following materials and methods were used throughout the work described here. Materials and methods specific to each study are described at the end of each chapter.

Cell Culture and Reagents.

Immortalized mouse embryonic fibroblasts (MEFs) were cultures in DMEM supplemented with 10% bovine calf serum, 1% penicillin and streptomycin, and 1% L-glutamine at 5% CO_2, 37°C. Cells were culture in 37 °C incubator with 5% CO_2. Cells treated with cycloheximide (Calbiochem) were used at 10 μg/mL in 100% EtOH. For proteasome inhibition, 10μM MG132 (Sigma) was used. Plat-e cells were grown in DMEM supplemented with 10% fetal bovine serum, 10μg Blasticidin , 1μg Puromycin when cultured during virus production were grown in DMEM supplemented with 10% fetal bovine serum % penicillin and streptomycin, and 1% L-glutamine.

Production of Virus

Plat-e cells (Morita et al., 2002) were transiently transfected with PEI (Reed et al., 2006) with 10 μg of pBabe and allowed to grow for 48 hours. Filtered virus was placed onto target cells along with 4μg/mL Polybrene (Sigma). Infected cells were selected with 2.5-μg/mL puromycin (Sigma).

SDS-PAGE.

After treatment with cycloheximide, cells were lysed in RIPA buffer [50mM Tris (pH 7.5), 150mM NaCl, 1%TritonX100, 1%NaDOC,.1%SDS, 1mM EDTA]. Whole cell extracts were prepared in lysis buffer and total protein concentrations were

normalized to each other using Lowry assay (BioRad) before immunoblot analysis. Cell extract was separated on a 4-15% gradient SDS-PAGE and transferred to PVDF membrane. IκBα was probed with either sc-371 (Santa Cruz Biotechnology) or sc-203, GFP was probed with sc-996, HA was probed with 16b12 (Covance), IκBε was probed with sc-7155, IκBβ – sc-945, β-actin – sc-1615. Quantification of immunoblots was performed with ImageJ and data analysis was performed with Kalidagraph (Synergy) or Excel (Microsoft). Dilution series with knockout extracts assured that Western blot signals were in the linear range.

Electrophoretic Mobility Shift Assay (EMSA)

After stimulation, cells were washed with ice cold Phosphate Buffered Saline (PBS) + 1mM EDTA, and were scraped and collected into a microcentrifuge tube and pelleted at 2000x*g*. Cells (about 10^6) were resuspended in 100ml CE Buffer [10mM HEPES-KOH (pH 7.9), 60mM KCl, 1mM EDTA, 0.5% NP-40, 1mM DTT, 1mM PMSF], and vortexed for lysis. Nuclei were pelleted at 4000x*g*, resuspended in 30ml NE Buffer [250mM Tris (pH 7.8), 60mM KCl, 1mM EDTA, 1mM DTT, 1mM PMSF], and lysed by 3 freeze-thaw cycles. Nuclear lysates were cleared by 14000x*g* centrifugation and protein concentrations were normalized via Bradford assay. 2.5ml total nuclear protein was reacted at room temperature for 15 minutes with 0.01 pmol of P^{32}-labeled 38bp double-stranded oligonucleotide containing two consensus kappaB sites: (GCTACAA**GGGACTTTCC**GCTG**GGGACTTTCC**AGGGAGG) in binding buffer [10mM Tris-Cl (pH 7.5), 50mM NaCl, 10% glycerol, 1% NP-40, 1mM EDTA, 0.1mg/ml polydI:dC], for a total reaction volume of 6ml. Complexes were resolved on a non-denaturing 5% acrylamide (30:0.8) gel containing 5% glycerol and 1X TGE

[24.8mM Tris, 190mM glycine, 1mM EDTA], and were visualized/quantitated using a PhosphorImager (Molecular Dynamics), in which the unbound probe (>20 fold excess) was used to normalized for loading variability.

Computational Simulations

The web interface mathematical model of the IκB-NFκB signaling module (http://signalingsystems.ucsd.edu/models-and-code/) was used (version 1.2; Kearns et al., 2006) for all simulations. Specific parameter changes are noted in Chapter 3.

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