Real-time dynamics of IκBα degradation studied with Kusabira-Orange 2 fusion proteins

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Preface

The nuclear factor-κB (NF-κB) consists of a family of transcription factors, which regulates diverse biological processes, including cell division, apoptosis and many aspects of immunological functions. In resting cells, NF-κB is stabilized by association with its inhibitors of IκB family, being IκBα the major inhibitor protein. NF-κB activation is initiated by a variety of stimuli such as pathogen associated molecules, cytokines and growth factors, which lead to activation of IκB kinase complex (IKK). IKK in turn phosphorylates IκBα, resulting in its degradation via the ubiquitin-mediated proteolytic pathway.

Pathological dysregulation of NF-κB is linked to inflammatory and autoimmune diseases as well as cancers. In these conditions IκBα is being continuously degraded. Although much has been learned about the biochemistry of IκBα degradation, it is unclear what factors control the kinetics and endurance of this process. In order to address this, it is of practical importance to develop sensitive tools to monitor the signal-induced degradation of IκBα in real-time in living cells.

Here, I proposed to develop fusion proteins of IκBα and monomeric fluorescent protein Kusabira-Orange 2 (mKO2) to visualize the dynamic behavior of IκBα degradation in real-time. The technique that has been described should provide a range of possible applications, for example the analysis of the dynamics and biochemical characteristics of IκBα degradation as well as for drug screening of potential proteasomal inhibitors. Moreover, it could be very valuable for analysis of protein-protein interactions in solution using mKO2 fusion proteins.

In this thesis, therefore, I constructed genetically encoded fusion proteins of full-length or fragments of human IκBα and mKO2 linked to its N terminus. Then, I have applied fluorescence based imaging to investigate the kinetics of the IκBα degradation in living cells. I checked the potential of this tool in the evaluation of inhibitors targeting ubiquitin-proteasomal pathway. Finally, I used computational approach to generate models and constructed detailed molecular dynamics simulations of fusion proteins in explicit solvent to investigate the structure-activity relationship of the degradation process.

This dissertation consists of three parts. First, construction of recombinant vectors and *in vitro* evaluation of fusion proteins are shown in Chapter I. In Chapter II, kinetics of IκBα degradation is investigated *in vitro* using HeLa cells. In Chapter III, structural properties of fusion proteins are analyzed using computational methods.

Studies in this dissertation demonstrate the development of fluorescent IκBα fusion proteins and their application to investigate the degradation and kinetics of IκBα in living cells as well as potential of this platform to be used as first-pass screening tool for inhibitors targeting NF-κB pathway. This work is to the best of our knowledge original, except where acknowledgements and references were made to previous works. Neither this, nor any other considerably similar work has been or is being considered to any other degree or diploma at any other institution.

Background and Strategy

The NF-κB transcription factor controls a range amount of target genes that play important role in cell survival, inflammation and immune system [1-3]. Initially NF-κB was described as a DNA-binding protein with affinity to the kappa immunoglobulin-light chain enhancer in B cells [4]. Later studies revealed that NF-κB activity is induced in almost all mammalian cell types and is involved in cellular responses to wide variety of stimuli, with important roles in cell division, regulation of apoptosis, growth factors and cytokine production, immune and inflammatory reactions [5, 6]. In mammalian cells, NF-κB signaling system consists of homo- or heterodimers comprising at least five members – RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). All NF-κB family members share a highly conserved Rel homology domain (RHD) that is responsible for binding to κB-site consensus sequence. In the resting cells, NF-κB proteins are retained inactive in the cytosol by binding to inhibitory proteins known as IκB, which are composed of multiple ankyrin (ANK) repeat domains [7, 8]. Most of IκB proteins have 6-7 ANK repeats that each consists of 33 amino acid residues and forms antiparallel α-helices separated by a loop. This family of proteins include cytoplasmic canonical IκB proteins, known as IκBα, IκBβ and IκBε, which are present in the cytoplasm of unstimulated cells and undergo stimulus-induced degradation; precursor proteins, p100 and p105, which can form the NF-κB family members p52 and p50, respectively, through their N-terminal segments; and nuclear IκBs IκBζ, Bcl-3 and IκBNS, which are absent in resting cells and induced upon cell stimulation [9, 10].

Classical (or canonical) NF-κB signaling pathway is shown in **Fig. 1**. Many different stimuli, such as pro-inflammatory cytokines, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), or Toll-like receptor (TLR) ligands activate the classical NF-κB pathway and recruit receptor proximal adaptor proteins signaling to IκB kinase complexes (IKK).

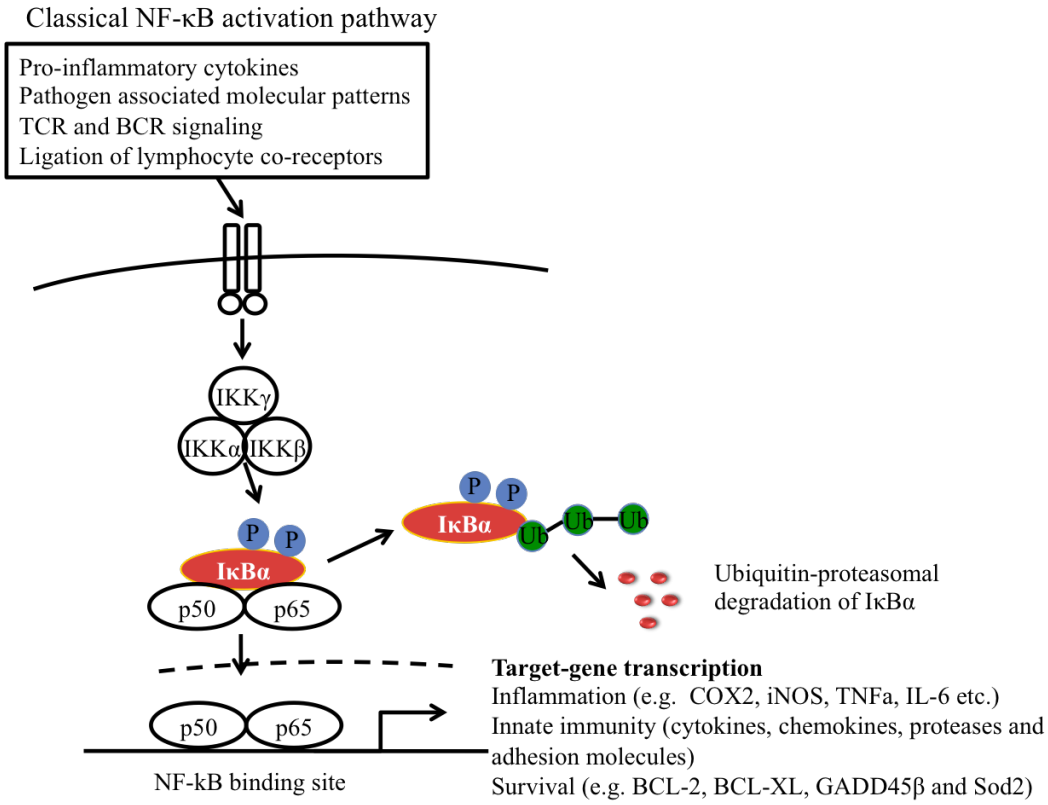

Fig. 1. Classical (or canonical) NF-κB signaling pathway. NF-κB dimers such as p50/p65 are maintained in the cytoplasm by interaction with inhibitory IκB molecules (often IκBα). Binding of a ligand to a cell surface receptor (e.g., TNFα or a TLR) recruits adaptors (e.g., TRAF and RIP) to the cytoplasmic domain of the receptor. In turn, these adaptors recruit an IKK complex onto the cytoplasmic adaptors (e.g., by ubiquitin-binding activity of NEMO). Activated IKK then phosphorylates IκBα at two serine residues, which leads to its ubiquitination at two lysine residues and degradation by the proteasome. NF-κB then translocates to the nucleus to activate target genes including IκBα for auto-regulation.

IKK (essentially through IKKβ) in turn phosphorylates IκBα at serine 32 and 36, leading to polyubiquitination of lysine 21 and 22 by β-TrCP containing Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 complexes and subsequent degradation by the 26S proteasome [11-13]. IκBα degradation unmasks nuclear localization segment (NLS) of NF-κB and allows released dimers to translocate into the nucleus and activate target genes [14]. Classical RelA/p50 heterodimers are predominantly regulated by IκBα.

IκBα regulation on NF-κB transcription factor is very critical and misregulation of these proteins results in many different diseases [15]. For example, continuous activation of NF-κB is observed in many types of cancers and malfunction of IκBα is associated

with Hodgkin's lymphomas [16].

IκBα is composed of a N-terminal region where the phosphorylation and ubiquitination occur; ANK repeats, which bind to NF-κB, and C-terminal PEST sequence rich with proline, glutamic acid, serine and threonine residues [17, 18]. Moreover, this acidic PEST motif has been shown to be crucial for interactions with NF-κB dimer and its subsequent removal from DNA [19-21]. ANK 5 and 6 of IκBα are weekly folded and very dynamic, but they fold once bound to NF-κB [22-24]. In contrast to stability in NF-κB-bound state, free IκBα is intrinsically unstable protein with half-life of less than 10 min. It was shown that basal degradation pathways of both free and bound IκBα occur through the same signaling pathway without IKK phosphorylation, the C-terminal PEST sequence or poly-ubiquitination [25]. However, contrary to these findings, another study showed that degradation of bound IκBα did not require IKK phosphorylation but require ubiquitination. Moreover, this work demonstrated that ubiquitination is necessary for the degradation of free IκBα [26]. Free IκBα turnover was demonstrated to involve casein kinase II mediated phosphorylation preferentially at C-terminal serine 293 and subsequent ubiquitination [27], but others reported that this occurs in signal-induced degradation of bound IκBα [28] and ubiquitination is not required [29, 30]. These distinct degradation mechanisms for free and bound IκBα appear to be critical for signal-induced NF-κB activation. Giving these contradictory results in the literature and the biological significance of IκBα, I aimed to construct fusion proteins comprising full-length or fragments of human IκBα and fluorescent protein mKO2 (mKO2-IκBα) to study signal-induced degradation kinetics of IκBα. I also generated fluorescent fusion proteins with mutant IκBα to identify the involvement of different regions of IκBα such as particular ANK repeats or PEST sequence in degradation process.

Fluorescent fusion protein technology and live cell imaging have provided the opportunity to observe intracellular events, to obtain spatial and temporal information of organelles and molecules, to conduct non-invasive assessments of molecular dynamics in living cells. It also allows for quick screening of new biological drug candidates. Recently, several works have utilized fluorescent proteins to track and report the dynamics of NF-κB. Pro-inflammatory stimuli such as TNFα or hydrogen peroxide caused GFP labeled p105 to translocate to the nucleus in 20 minutes [31]. Fusion protein

of IκBα-EGFP was shown to be rapidly degraded upon TNFα or phorbol myristate acetate triggered NF-κB activation. Fluorescence resonance energy transfer (FRET) was used to analyze the interaction between p65 and IκBα using GFP proteins with dual fluorescence properties [32]. EGFP labeled p65 fusion construct was used to investigate the kinetics of NF-κB pathway in living cells upon the IL-1β stimulation [33]. The expression levels of p65-EGFP was influencing factor for the kinetics of the response to the IL-1β stimulation as well as for the NF-κB-promoted anti-apoptotic effect. This fusion construct in addition to IκBα was also used to investigate the shuttling mechanism of these proteins between nucleus and cytoplasm [34]. Recent study applied fluorescence imaging of p65 and IκBα in combination with luminescence imaging of NF-κB transcription process to investigate real-time kinetics of the NF-κB dependent transcription. Recently, Sakaue-Sawamo et al. fused fluorescent protein with the optimal fragment of the cyclin-dependent kinase inhibitor p27, which degrades through ubiquitin-proteasome pathway in cell cycle dependent manner [35]. They demonstrated that the fusion protein degraded in cell cycle dependent manner. Although much has been clarified about the biochemistry of this signaling pathway, it is unclear what factors are responsible to the kinetics and endurance of the degradation process. To address this, it is of practical importance to develop sensitive tools to visualize the signaling pathway in real-time in living cells. In addition, I used computational approach to evaluate the effect of IκBα mutations in NF-κB binding and degradation.

Chapter I

Design and construction of fusion proteins composed of fluorescent mKO2 and IκBα variants

I.1 Conclusion

Novel constructs to monitor the behavior and dynamics of IκBα protein were developed. The strategy involves the construction of six novel expression vectors, in which the mKO2 gene was fused to full-length or fragments of human IκBα. Three domains of IκBα, i.e., N-terminal phosphorylation and ubiquitination domain, ANK repeats 1 and 2, and C-terminal PEST domain, were fused to mKO2 with amino acid linker $(GGGGS)_3$ (GS linker) in different combinations to construct mKO2::hIκBα, mKO2::IκBα140(IκBαΔANK3-6,PEST), mKO2::IκBα74(IκBαΔANK1-6,PEST), mKO2::IκBα140-PEST(IκBαΔANK3-6), mKO2::IκBα74-PEST(IκBαΔANK1-6) and mKO2::IκBα277(IκBαΔPEST). Results of western blot and sequencing confirmed the expression of these vectors. All constructed vectors were confirmed to be effectively expressed *in vitro* in transfected HeLa cells as demonstrated by the appearance of the fluorescence signal of mKO2. The constructed fusion proteins were suggested to serve as the potential tool for analysis of IκBα protein dynamics.

Chapter II
Real-time imaging and analysis of degradation kinetics of IκBα using mKO2 fusion proteins in living cells

II.1 Conclusion

Real-time confocal microscopy and western blot analysis showed that, like endogenous IκBα, fusion-proteins expressed in HeLa cells degraded after TNFα stimulation. Decrease of fluorescence intensity was observed at approximately 5-7 minutes post-stimulation with TNFα. In this *in vitro* setting, degradation dynamics of recombinant probes were comparable to those observed with endogenous IκBα. No decrease in fluorescence was observed in the cells transfected with mKO2 alone, suggesting that degradation of fluorescent proteins occurs only if fused to IκBα. Fusion proteins lacking the PEST motif exhibited no stimuli-induced degradation, as monitored by time-laps fluorescence imaging. From confocal microscopy data obtained for fusion proteins with mutations at PEST site, it is concluded that PEST sequence is essential in signal-induced degradation of IκBα and functions independently from ANK repeats. Proteasome inhibitors such as lactacystin and MG132 blocked the decay of fluorescence in all PEST-containing degradable probes, suggesting that degradation of fusion proteins in stimulated cells occurs via proteasomal system. The degradation rate of mKO2::IκBα74-PEST(IκBαΔANK1-6) was smaller than mKO2::IκBα140-PEST(IκBαΔANK3-6) and mKO2::IκBα proteins, which in turn was similar to the half-life of native IκBα.

Chapter III

Structure-activity relationship of fusion proteins: an *in silico* analysis

III.1 Conclusion

The three-dimensional structures of mKO2::IκBα, mKO2::IκBα74-PEST(IκBαΔANK1-6) and mKO2::IκBα140-PEST(IκBαΔANK3-6) were modeled using comparative (homology) modeling approach. Subsequently, molecular dynamic simulations of these proteins in the presence of explicit water were performed to optimize the models and assess the stability. The confidence of the models was quantified based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. Models of mKO2::IκBα (C-score=-0.95, RMSD=9.8±4.6Å), mKO2::IκBα74-PEST(IκBαΔANK1-6) (C-score=-0.06, RMSD=6.7±4.0Å) and mKO2::IκBα140-PEST(IκBαΔANK3-6) (C-score=-1.03, RMSD=9.3±4.6Å) were of correct global topology. ANK repeats of the designed models were described as two anti-parallel α-helices, followed by a loop at nearly right angle stabilized by the hydrophobic interactions within the repeats. In all constructs, GS-linker allowed the mKO2 to keep distance from functional domains and N-terminal region of IκBα to be accessible for both phosphorylation and ubiquitination. Furthermore, the refined models by molecular dynamics were used for multiple protein-protein docking studies for the identification of their complexes with p65/p50 NF-κB subunits in order to study the effect of fusion construct to the stability of the complex as well as effect of mutations in binding and degradation processes. Fusion proteins did not create a single continuous buried surface contact with NF-κB but rather interacted through several independent smaller patches. mKO2::IκBα protein bound to NF-κB in a similar mode as IκBα by masking NLS segment of p65. Displacement of mKO2::IκBα140-PEST(IκBαΔANK3-6) resulted in increased solvent accessibility for phosphorylation domain Ser32 that resulted in faster degradation. Solvent accessibility of both ubiquitination residues Lys21 and Lys22 of mKO2::IκBα74-PEST(IκBαΔANK1-6) decreased resulting in slower degradation.

Summary

Novel fusion proteins composed of fluorescent protein mKO2 and human IκBα or its fragments were developed to evaluate dynamics of stimulus-triggered degradation of IκBα in living cells. Real-time confocal microscopy analysis showed that fusion-proteins expressed in HeLa cells degraded after TNFα stimulation. The half-life of degradation of mKO2::IκBα74-PEST(IκBαΔANK1-6) was smaller than mKO2::IκBα140-PEST(IκBαΔANK3-6) and mKO2::IκBα proteins, which in turn was similar to the native IκBα. Mutation studies demonstrated that PEST sequence is essential in signal-induced degradation of IκBα independently from ANK repeats.

Selective proteasome inhibitors blocked the decay of fluorescence in concentration dependent manner, suggesting that stimuli-induced degradation of fusion proteins occurs via proteasomal system. Kinetics of degradation of these proteins obtained by real-time monitoring in the presence or absence of proteasome inhibitors upon the pro-inflammatory stimuli provided a reliable platform for the first-pass screening and evaluation of new drugs targeting the NF-κB pathway.

Molecular-modeling approach was used to obtain structural models and reveal the potential sites of interaction between fusion proteins and NF-κB as well as the effect of mutations in binding and degradation processes. The mKO2::IκBα bound to NF-κB similarly as native IκBα masking nuclear localization domain. In all proteins GS-linker kept mKO2 in a distance from phosphorylation and ubiquitination sites of IκBα to provide enough accessibility for the enzymes. Different binding schemes of partially modified IκBα fusion proteins with NF-κB altered surface accessible solvent area for phosphorylation and ubiquitination residues resulting in changed kinetics of degradation, which was consistent with microscopy data. The technique that has been described here could provide a range of possible applications, such as the analysis of the dynamics and biochemical characteristics of IκBα degradation as well as for screening of potential proteasomal inhibitors.

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