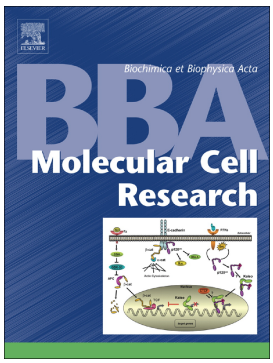

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Development of mKO2 fusion proteins for real-time imaging and mechanistic investigation of the degradation kinetics of human IκBα in living cells

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Abstract

In resting cells, the nuclear factor kappa B (NF-κB) family of transcription factors is stabilized by complexation with the cytoplasmic inhibitor of kappa B alpha (IκBα). Extracellular stimuli, such as tumor necrosis factor alpha (TNFα) or bacterial lipopolysaccharide activate NF-κB through IκBα phosphorylation and ubiquitin-proteasomal degradation. Herein, we developed a novel biosensor, by fusing the monomeric fluorescent protein Kusabira-Orange 2 to IκBα (mKO2-IκBα), to study the dynamics and structure-activity relationship of IκBα degradation. Site-specific deletion studies on the IκBα sequence revealed that the C-terminal PEST domain is required in signal-induced proteasomal degradation of IκBα and functions independently from ankyrin repeats. Using deletion mutants, we show that IκBα ankyrin repeats do not affect IκBα degradability but affect its degradation rate. We demonstrate, by both real-time confocal microscopy and western blot analysis, that the half-life of mKO2-IκBα in response to TNFα is approximately 35 min, which is similar to the half-life of endogenous IκBα. Using this biosensor we also show that selective proteasome inhibitors, such as lactacystin and MG132, inhibit degradation and affect the kinetics of IκBα in a dose-dependent manner. The techniques described here can have a range of possible applications, such as facilitating studies associated with IκBα dynamics and biochemical characteristics, as well as the screening of potential proteasome inhibitors.

Keywords: IκBα, Kusabira-Orange 2, Ubiquitin-proteasomal degradation, PEST domain, Ankyrin repeats

1. Introduction

The nuclear factor-κB (NF-κB) transcription factor controls a range of genes that play important roles in cell survival and in immune and inflammatory responses [1–3]. In resting cells, NF-κB proteins are retained inactive in the cytosol through binding to IκBα, the primary inhibitory protein [4,5]. IκBα is composed of a N-terminal domain, six ankyrin repeat domains (ANK), and an acidic C-terminal PEST sequence rich in proline, glutamic acid, serine, and threonine residues [6,7]. Many different stimuli, including cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), pathogen- and damage-associated patterns, and nucleic acids, activate the NF-κB pathway. Although these ligands utilize different NF-κB transduction mechanisms, they all activate the IκB kinase complexes (IKK). In turn, IKK (essentially through IKKβ) phosphorylates IκBα at serine residues 32 and 36, leading to the polyubiquitination of lysine residues 21 and 22 in its N-terminus, by β-TrCP-containing Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 complexes, and subsequent IκBα degradation by the 26S proteasome [8–10]. IκBα degradation exposes the nuclear localization segment (NLS) of NF-κB, which is predominantly stabilized by ANK1 and ANK2 [6,7], and allows the released dimers to translocate into the nucleus and activate target genes [11]. Some studies have suggested that the C-terminal PEST domain is required for signal-induced degradation of IκBα, as demonstrated in a cell-free system [12]. In contrast, others show that the PEST domain is not required for degradation [13]. Although the biochemistry of this signaling pathway has been intensively investigated, the structural factors that contribute to the initiation and duration of the degradation process have yet to be determined. To address these dynamics, non-invasive tools are required to measure the IκBα degradation in real-time in living cells.

IκBα-mediated regulation of NF-κB (mainly of the RelA/p50 heterodimer) is critical in cellular processes such as the immune response, cell growth, differentiation, and apoptosis [3,14–16], and its deregulation results in many diseases [17]. For example, continuous activation of NF-κB is observed in many types of cancers, and malfunction of IκBα is associated with Hodgkin's lymphomas [18]. Many groups have undertaken major efforts towards the development of selective inhibitors of different downstream levels of the NFκB pathway, such as IKK activity [19–22] and NFκB nuclear translocation [23]

and DNA-binding activity [24]. Much of this effort involved the screening of compounds that inhibit the 26S proteasome for the development of therapeutic agents to be used in the treatment of cancers, as well as of inflammatory diseases [25–27]. Indeed, inhibitors of the 26S proteasome have been shown to inhibit the degradation of IκBα and nuclear translocation of NFκB [28]. Therefore, real-time visualization of IκBα degradation should also provide cell-based assays for the development of more selective and efficient inhibitors of IκBα degradation, *e.g.* proteasome inhibitors.

In this study, we aimed to construct genetically encoded fusion proteins of human IκBα and monomeric Kusabira-Orange 2 fluorescent protein (mKO2), in which mKO2 is fused to the IκBα N-terminus, to investigate the kinetics of signal-induced degradation of IκBα in HeLa cells. Additionally, to examine how IκBα structural elements correlate with its signal-induced degradation, we also generated fluorescent probes containing deletions in IκBα ANK repeats and/or in its C-terminal PEST domain. Using these mutants, we identified the regions required for the degradation process. Furthermore, we investigated the potential of this platform to be used as a primary screening tool for inhibitors targeting the ubiquitin-proteasome pathway.

2. Materials and Methods

2.1. Vector construction

Fluorescent protein mKO2 (MBL Co. LTD., Japan) was PCR-amplified using primers containing *Nhe*I and *Kpn*I restriction sites (forward primer: GGAACCGCTAGCATGGTGAGTGTGATTAAACC; reverse primer: AGAAGATGCAGTAGCTCATTGGTACCGCGGTA), and the digested products were cloned into pcDNA3.1 vector (Thermo Fisher Scientific Inc., Rockford, IL) to generate pcDNA3.1-mKO2. The GS linker (GGGGSx3; PBS-coupler1, Riken Bioresource Center, Tsukuba, Japan) was amplified using primers containing *Kpn*I and *Xba*I restriction sites (forward primer: AAAGGTACCAACCCCTCGAGGTCGACGGTAT; reverse primer: TTGCTCTAGAACTAGTGGATCCCCCGGGCT), and the digested products were cloned in-frame into pcDNA3.1-mKO2 to generate pcDNA3.1-mKO2-GS. The full-length or fragments of human IκBα cDNA (Gene ID: 4792; Clone ID: RDB 6668; Riken) were amplified using the forward primer containing *EcoR*I,

AATTAAGAATTCTTCCAGGCGGCCGAGCGCC and the following reverse primers containing *BamH*I: TGTAGGATCCTGCACTCATAACGTCAGACGCTG for full-length IκBα, AGCAGCTCACCGAGGACGGGGGATCCATAATT for the first 74 aa of IκBα (IκBα74), and GCTGTGATCCTGAGCTCCGAGGATCCGCAT the first 140 aa of IκBα (IκBα140). The digested products were cloned in-frame into pcDNA3.1-mKO2-GS to generate pcDNA3.1-mKO2-GS-IκBα, pcDNA3.1-mKO2-GS-IκBα74, and pcDNA3.1-mKO2-GS-IκBα140. To clone the full-length or the fragments of human IκBα protein, the forward primer was designed without the start codon to ensure that the final protein was expressed as a fusion protein with mKO2 on its N-terminus. The PEST domain was amplified using primers containing *BamH*I and *Xba*I (forward primer: AATTGGATCCCAGATGCTGCCAGAGAGTGA; reverse primer: GCCAGCGTCTGACGTTATGATCTAGAGGAA), and the digested products were cloned into pcDNA3.1-mKO2-GS. To construct the vectors containing the PEST domain but having ANK1-6 or ANK3-6 domains deleted, the cDNA fragments of human IκBα were amplified using primers containing *Eco*RI and *BamH*I (forward primer: AATTAAGAATTCTTCCAGGCGGCCGAGCGCC: reverse primer: AGCAGCTCACCGAGGACGGGGGATCCATAATT, without the stop codon), and the digested products were cloned in-frame into pcDNA-mKO2-GS-PEST, between GS and PEST, to generate pcDNA3.1-mKO2-GS-IκBα74-PEST (lacking all ANK domains) and pcDNA3.1-mKO2-GS-IκBα140-PEST (lacking ANK3-6) probes. The human IκBα sequence corresponding to the first 277 aa without the PEST region (IκBα277) was amplified using primers containing *Eco*RI and *BamH*I (forward primer: AATTAAGAATTCTTCCAGGCGGCCGAGCGCC; reverse primer: GGCCGGATCCTTAAAGGTTTTCTAGTGTCAGCTGG, with an added stop codon) to generate pcDNA3.1-mKO2-GS-IκBα277. After transformation of bacteria with the constructs, colonies were screened by colony PCR and the DNA from positive clones was purified using the Promega MiniPrep Kit (Madison, WI), according to the manufacturer's protocol, and subjected to sequencing.

2.2. Cell culturing and transfection

For confocal and fluorescence microscopy, HeLa cells (Riken Bioresource Center, Tsukuba, Japan) were plated in 35 mm glass-bottom dishes (Iwaki, Japan), at 1×10^5 cells in 2 mL of DMEM supplemented with 10% FBS, at 37°C, with 5% CO_2. After 24 h, cells were washed three times in PBS, and the medium was replaced by Opti-MEM I® reduced-serum medium (Life Technologies Inc., Gaithersburg, MD) and transfected with the plasmids, using FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions [transfection reagent (µL):pDNA (µg) = 3:1]. Forty-eight hours after transfection, cells were washed three times in PBS, and the medium was replaced with fresh Opti-MEM I® without phenol red. Cells were stimulated with 10 ng/mL TNFα (MilliporeSigma, Allentown, PA) and subjected to live-cell imaging. To evaluate protein expression, 48 h after transfection, cells were washed three times in PBS, fixed in 4% paraformaldehyde, at room temperature, for 15 min, and imaged by microscopy.

2.3. Fluorescence microscopy

Time-lapse confocal microscopy was carried out on transfected cells in 35 mm glass-bottom dishes in the Nikon Eclipse Ti inverted microscope equipped with A1R MP Multiphoton Confocal system (Nikon Instruments Inc., Tokyo, Japan), in humidified conditions, at 37°C, 5% CO_2, and using 10× or 20× Plan Apo objective lenses (Nikon Instruments Inc., Tokyo, Japan). Excitation of mKO2 was performed at 551 nm. Data were recorded at different time frames, and processing was carried out using built-in NIS Elements AR acquisition and analysis software (Nikon Instruments Inc., Tokyo, Japan). For fusion proteins, mean fluorescence intensities were calculated at each time-point per cell, and the respective fluorescence intensity relative to the starting fluorescence signal was obtained. After subtracting the background signal, the fluorescence intensity of the cells pixels in each image was converted to inherent values for each individual cell, using ImageJ 1.48v software (National Institutes of Health, MD, USA). The results are expressed as the mean ± S.D. of at least three independent cell cultures. Half-life values were obtained from the curve fitting the data to the decay function, using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Fluorescent microscopy of fixed cells was carried out using Biozero BZ-8000 microscope (Keyence, Osaka, Japan).

2.4. Western blot

HeLa cells were grown in a 100-mm culture dish in DMEM supplemented with 10% FBS. When the confluence was reached, the medium was replaced by Opti-MEM I, and cells were transiently transfected as described above. Forty-eight hours after transfection, cells were stimulated with 10 ng/mL TNFα for the indicated times and, then, washed with PBS. Extraction of cytoplasmic proteins was performed using Active Motif Nuclear extraction kit (Active Motif, Carlsbad, CA), and the extract was quantified using Bradford Assay kit (Bio-Rad, Hercules, CA), according the respective manufacturers' instructions. The cytoplasmic fraction was diluted in 2× SDS-PAGE loading buffer (0.125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue) and denatured at 90°C for 5 min. Proteins were separated by polyacrylamide gel electrophoresis, using 12.5% SuperSep Ace (Wako Pure Chemical Industries LTD., Osaka, Japan) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked overnight, at 4°C, in 3% BSA containing 1% Tween 20, and probed using anti-IκBα (sc-847, Santa Cruz), anti-IκBα c-21 (sc-371, Santa Cruz), anti-mKO2 (PM051, MBL), and anti-β-actin-HRP (sc-47778, Santa Cruz) antibodies. Proteins were detected with goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz). Chemiluminescence was developed using Luminata Western Chemiluminescent HRP Substrate (Millipore, Sigma). Densitometric analysis was performed using ImageJ 1.48v software, and the specific bands were quantified based on their relative intensities to β-actin.

2.5. Treatment of cells with TNFα and proteasome inhibitors

In all experiments, cell cultures were kept in DMEM containing 10% FBS for 24 h before the stimulations. Cells were treated with TNFα immediately before microscopy analysis, by replacing 1/10 of the culture medium volume in the 35-mm culture dish with TNFα to a final concentration of 10 ng/mL. Cells were treated with lactacystin for 24 hours prior TNFα treatment, at a concentration of 25 μM, except where indicated otherwise. DMSO (1%, v/v) was used as vehicle for lactacystin and was run in parallel as control in all cases. A series of experiments using another inhibitor of the proteasome,

MG132, were performed to compare with the lactacystin results. The concentration of MG132 used was 50 μM, and the control containing 0.25% (v/v) DMSO (the solvent used as vehicle) was performed. Non-treated and vehicle-treated cells yielded similar results. Each experiment was carried out at least three times, with at least 10-15 cells per repeat.

3. Results and Discussion

3.1. Development of degradable full-length and partially modified fusion mKO2-IκBα proteins

The red fluorescent protein mKO2 was fused to full-length and to fragments of human IκBα protein to develop fluorescent probes to investigate the stimulus-induced degradation of IκBα. Three domains were chosen as fragments of IκBα: the N-terminus phosphorylation-ubiquitination domain, the ANK repeats 1 and 2, and the C-terminal PEST domain. The mKO2 protein was fused to the N-terminus phosphorylation-ubiquitination domain via an GS linker in three different combinations: 1) mKO2 was fused to the full-length human IκBα (317 aa) to obtain the mKO2::IκBα probe; 2) a 140-aa fragment of IκBα containing the N-terminus phosphorylation-ubiquitination domain and ANK1 and ANK2 was fused to mKO2 to yield the mKO2::IκBα140(IκBαΔANK3-6,PEST) probe (this fusion protein lacks ANK 3,4,5, and 6 and the C-terminal PEST region of IκBα), and 3) ANK1 and ANK2 were deleted, and the N-terminus of IκBα (74 aa), containing only the phosphorylation-ubiquitination domain, was fused to mKO2 to obtain the mKO2::IκBα74(IκBαΔANK1-6,PEST) probe (this fusion protein lacks all six ANK repeats and the C-terminal PEST region of IκBα) (**Fig. 1a**). We observed that proteins mKO2::IκBα140(IκBαΔANK3-6,PEST) and mKO2::IκBα74(IκBαΔANK1-6,PEST) failed to degrade upon TNFα stimulus in HeLa cells, as shown and discussed later.

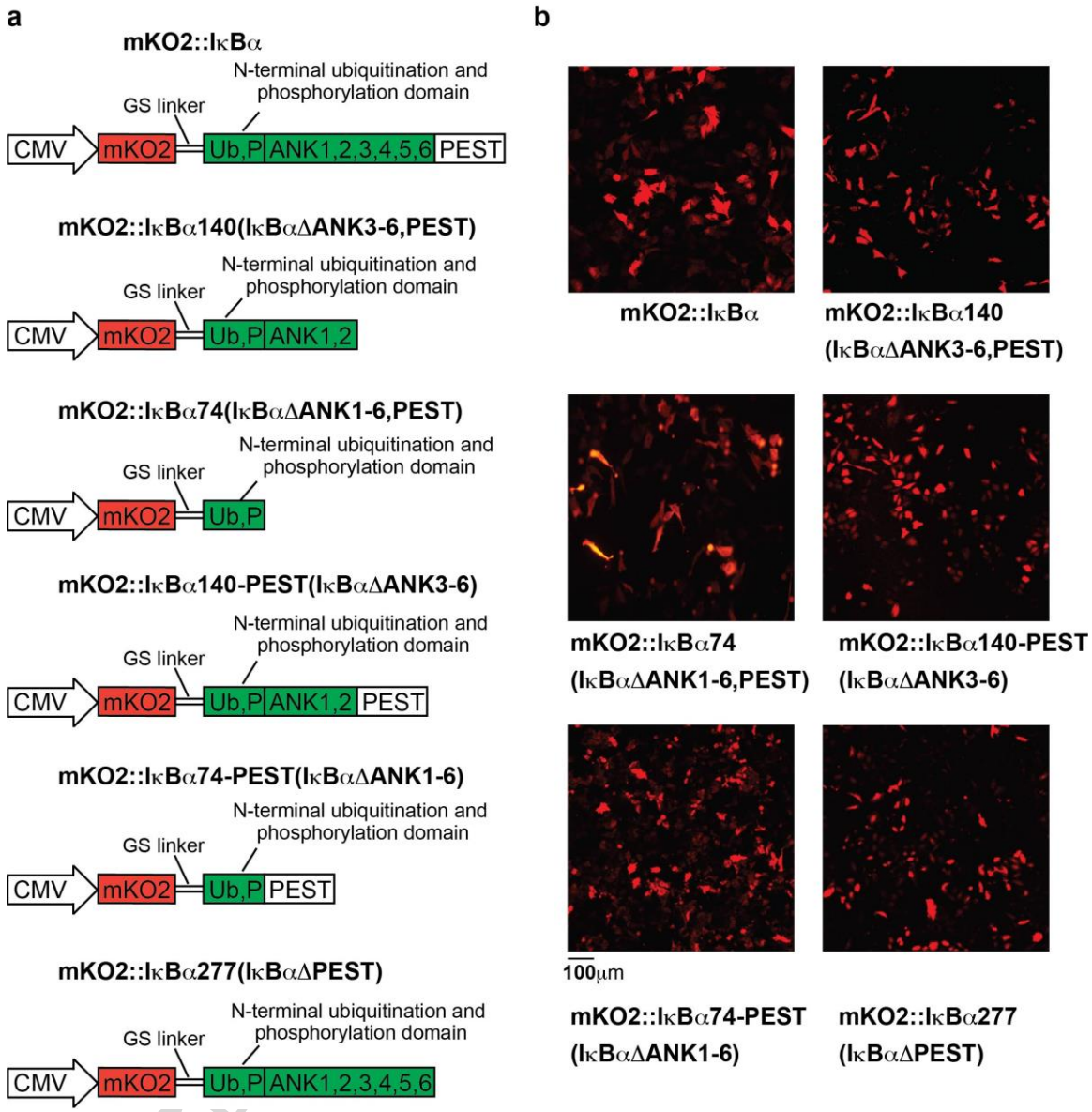

Fig. 1. Vector design and expression of fusion proteins. (a) Plasmid vector constructions. CMV: cytomegalovirus promoter for the transcription of mKO2; mKO2: monomeric Kusabira orange 2 gene; Ub, P: ubiquitination and phosphorylation domain; ANK: ankyrin repeat domain; PEST: sequence of proline, serine, threonine, aspartate, and glutamate residues. **(b)** Fluorescence images of HeLa cells expressing IκBα fusion proteins. 1×10^5 HeLa cells were transfected with the indicated plasmids. Two days after transfection, cells were fixed and imaged by fluorescence microscopy.

We hypothesized that PEST might be required independently from ANK repeats for stimulus-induced degradation. To test this hypothesis, three deletion mutants of IκBα

were generated: 1) all six ANK repeats were deleted, and the PEST sequence was directly fused to the phosphorylation-ubiquitination domain of IκBα (aa 1-74) to produce mKO2::IκBα74-PEST(IκBαΔANK1-6); 2) ANK3-6 was deleted, and the PEST domain was fused to IκBα containing the phosphorylation-ubiquitination domain and ANK1 and ANK2 (aa 1-140) to obtain mKO2::IκBα140-PEST(IκBαΔANK3-6), and 3) the 1-277 aa fragment of human IκBα was fused to mKO2 to generate mKO2::IκBα277(IκBαΔPEST) probe, *i.e.* the full-length IκBα lacking the PEST domain (**Fig. 1a**). The constructed vectors were transiently transfected into HeLa cells, followed by fixation and imaging by fluorescence microscopy. All fusion proteins expressed well in transfected cells, as confirmed by fluorescence microscopy (**Fig. 1b**). In non-stimulated cells expressing the fusion proteins, a strong red fluorescence signal distributed throughout the cytoplasm was readily observed. The strongest intensity was obtained with a transfection efficiency above 60%, when cells were kept at 37°C for 24 h after removing the transfection complex and, then, incubated at 37°C, for another 24 h.

3.2. Visualization of TNFα-induced IκBα degradation in HeLa cells

Exposure of cells to a pro-inflammatory stimuli, such as TNFα, leads to the rapid degradation of IκBα by a proteolytic system that is required for nuclear translocation and activation of NF-κB [8–10]. To continuously visualize the degradation of IκBα in the cytosol, we used fusion proteins and studied them in transfected HeLa cells by confocal microscopy. Live-cell imaging was performed within a 2-h time-frame after TNFα stimulation. In time-lapse images of cells transfected with mKO2::IκBα (*i.e.*, fusion with the full-length human IκBα protein), fluorescence intensity began to decay approximately 5-7 min after the addition of 10 ng/mL TNFα to the culture medium (**Fig. 2a** and **Supplementary movie 1**). To confirm that the decay in fluorescence correlated with a decrease in IκBα protein levels, we measured the amount of the fusion protein by western blot, using IκBα-specific antibodies. In the non-stimulated sample, we observed a band at 63 kDa, corresponding to the predicted size of the mKO2::IκBα protein (**Fig. 2b**). The intensity of the band declined with the increasing time of TNFα exposure and was reduced to approximately 50% after approximately 35 min of treatment, showing that

the half-life of the mKO2::IκBα protein is 35 min. This observation is in agreement with those reported previously for the endogenous protein (approximately 30 min) [13,29–32], and as found in our western blot data ($T_{1/2}$ = 34 min) (**Fig. 2c**). Therefore, the intracellular fluorescence intensity of the fusion protein directly correlates with the IκBα protein level. Similarly, quantitative analysis of time-lapse imaging demonstrated that decay of fluorescence occurred with the half-life of 37 min (**Fig. 2d**). No fluorescence decay was observed in cells expressing solely the mKO2 protein (*i.e.*, without being fused to IκBα), after TNFα stimulation (**Supplementary Fig. 1**).

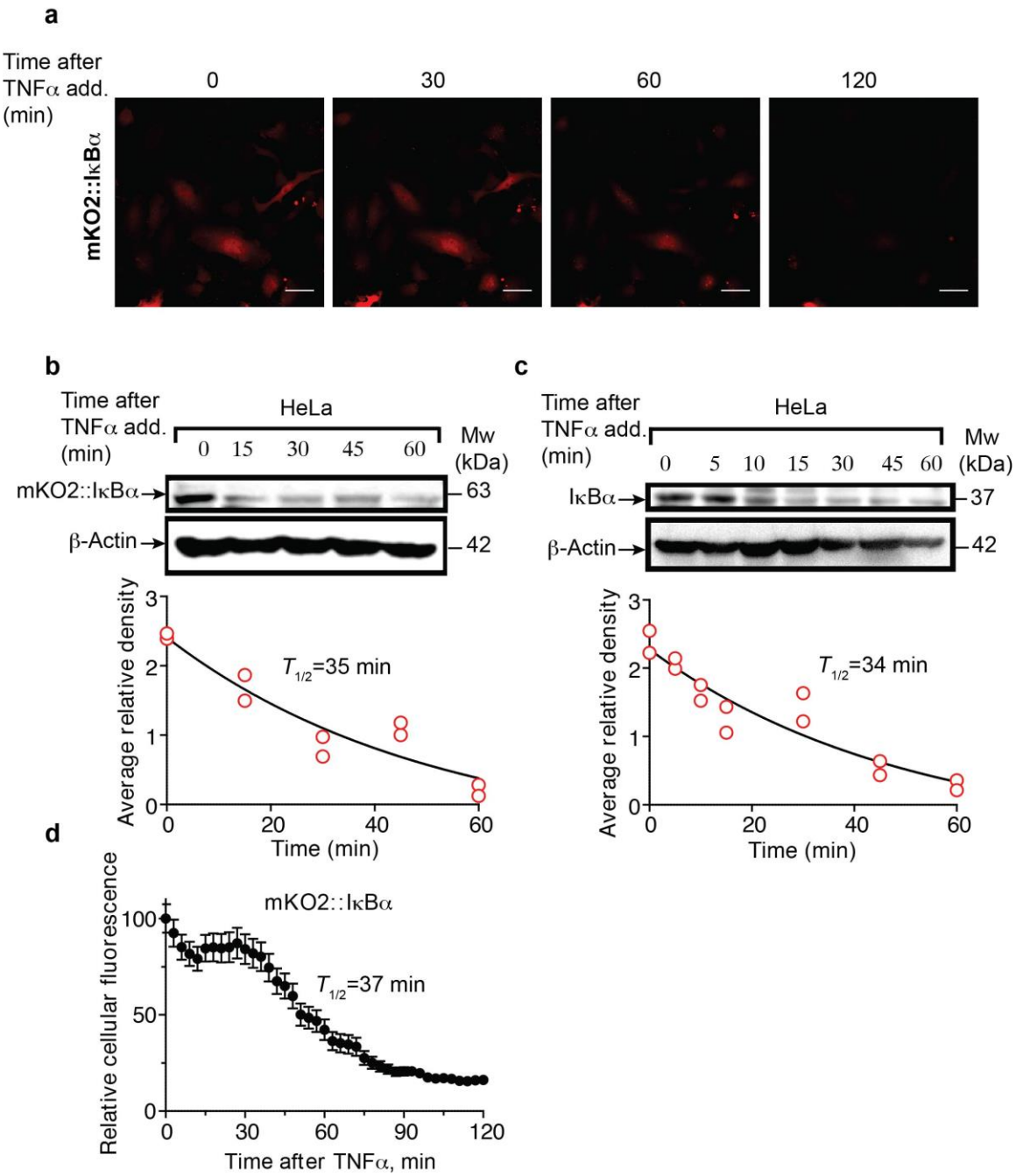

Fig. 2. **Degradation of mKO2::IκBα in response to TNFα stimulation. (a)** Time-lapse images of mKO2::IκBα fluorescence at indicated times (min) after addition of TNFα (10 ng/mL). Scale: 50 µm. **(b, c)** Western blot analysis and quantitative densitometry of the degradation of **(b)** mKO2::IκBα and **(c)** endogenous IκBα protein levels in HeLa cells stimulated with 10 ng/mL TNFα for the indicated times. Selective bands were quantified based on their relative intensities to β-actin. **(d)** Quantitative analysis of mKO2::IκBα fluorescence decay. Transfected HeLa cells were stimulated with 10 ng/mL TNFα and monitored for 2 h. The mean cellular fluorescence

intensities were determined at each time-point and plotted as a percentage of the fluorescence at t = 0 min. Data are shown as the mean ± SD from three experiments, with at least 10 cells per experiment.

3.3. Involvement of ANK repeats in TNFα-induced IκBα degradation

It is well established that the ANK repeats are required for the association of IκBα with NFκB [3,7,14,33]. However, it is not clear whether these residues contribute directly to IκBα degradation. To understand this, we generated IκBα deletion mutants by removing its ANK repeats and/or C-terminal PEST sequence. Amino acid residues vicinal to both p50 and p65 NLS are restrained to the first two ANK repeats of IκBα, suggesting a mechanism for cytoplasmic retention of NF-κB [6]. First, we deleted all ANK repeats and the C-terminal PEST domain, leaving only the N-terminal phosphorylation-ubiquitination domain [mKO2::IκBα74(IκBαΔANK1-6,PEST)]. There was no decay in the fluorescence in response to TNFα in cells expressing this probe (**Fig. 3**). It should be noted that since this probe does not contain ANK repeats, it most likely does not associate with NFκB and is free in the cytosol. Next, we deleted ANK3-6 repeats [mKO2::IκBα140(IκBαΔANK3-6,PEST)]. This probe did not degrade upon TNFα stimulation, either (**Fig. 3**), suggesting that the region responsible for degradation lies downstream the ANK repeats towards the C-terminus. To test this hypothesis and investigate how ANK 3-6 repeats can potentiate the signal-induced degradation of IκBα, cells were transfected with the vector carrying mKO2 fused to full-length IκBα lacking the C-terminal PEST domain [mKO2::IκBα277(IκBαΔPEST)] (*i.e.* mKO2 fused to the N-terminal and all six ANK domains). The mKO2::IκBα277(IκBαΔPEST) was not degraded upon stimulation with TNFα (**Fig. 3**). This result suggests that, *per se*, ANK repeats do not have an impact on the degradability of IκBα.

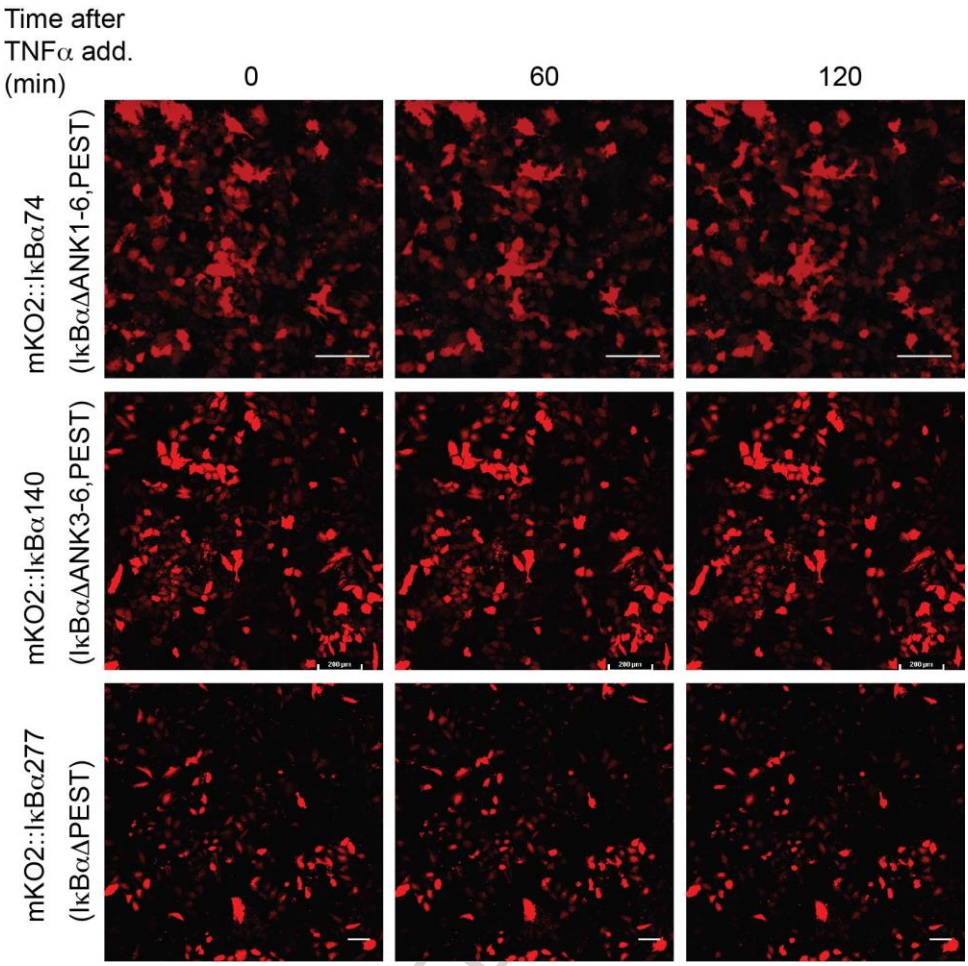

Fig. 3. Time-lapse confocal microscopy of IκBα ANK deletion mutants in response to TNFα. HeLa cells (1×10^5) were transfected with the indicated plasmids. Two days after transfection, cells were stimulated with 10 ng/mL TNFα and monitored for 2 h. Scale: 200 μm. No degradation was observed in cells expressing the mKO2::IκBα74(IκBαΔANK1-6,PEST), mKO2::IκBα140(IκBαΔANK3-6,PEST), or mKO2::IkBa277(IκBαΔPEST) fusion proteins.

3.4. The role of the PEST region in TNFα-induced IκBα degradation

Our results regarding real-time degradation analysis of PEST-deleted mutant probes, *i.e.*, mKO2::IκBα140(IκBαΔANK3-6,PEST), mKO2::IκBα74(IκBαΔANK1-6,PEST), and mKO2::IκBα277(IκBαΔPEST), imply that the PEST sequence may be required for stimulus-triggered degradation of IκBα. It has been previously demonstrated that deletion of entire C-terminal of IκBα does not affect its binding to NFκB and C-terminal does not contact the NLS sequence [34]. Therefore, we generated two fusion proteins by cloning the 40-aa PEST domain directly into the N-terminal phosphorylation-

ubiquitination domain after removing all six ANKs [mKO2::IκBα74-PEST(IκBαΔANK1-6)], and into the ANK 1-2 segment [mKO2::IκBα140-PEST(IκBαΔANK3-6)] that responsible for masking the NLS. As a result, a clear decay in fluorescence was observed in cells expressing mKO2::IκBα74-PEST(IκBαΔANK1-6) or mKO2::IκBα140-PEST(IκBαΔANK3-6) from approximately 5-7 min post-TNFα stimulation onwards (**Fig. 4a** and **Supplementary movies 2** and **3,** respectively). Although there are contradictory reports on the involvement of the PEST sequence in IκBα degradation [12,13,35], our data support that PEST is necessary for the signal-induced IκBα degradation. Moreover, we show that the PEST sequence functions independently from ANK repeats. Stimulus-induced degradation of mKO2::IκBα74-PEST(IκBαΔANK1-6) and of mKO2::IκBα140-PEST(IκBαΔANK3-6) was also confirmed by western blot analysis of cytoplasmic extracts using specific anti-mKO2 antibodies (**Fig. 4b**). In transfected cells expressing mKO2::IκBα74-PEST(IκBαΔANK1-6), degradation reached 50% of the initial cellular fluorescence in 50 min. The half-life of mKO2::IκBα74-PEST(IκBαΔANK1-6) was consistent with the previously observed half-life of free IκBα (45-60 min) [31,36]. It has previously been shown, using insect cells, that IκBα in association with NFκB is more efficiently phosphorylated and degraded than free IκBα [37]. It is also known that IKK is not controlling the degradation of free IκBα in cytoplasm [38]. This may explain why newly synthesized IκBα, upon stimulation, is not immediately phosphorylated and degraded in cells. Since mKO2::IκBα74-PEST(IκBαΔANK1-6) probe lacks all ANK repeats and is not likely to form a complex with NFκB, it is less favorable for phosphorylation and ubiquitination. Therefore, it can be precluded that longer retention of mKO2::IκBα74-PEST(IκBαΔANK1-6) probe is the result of ubiquitin-independent degradation pathway. Indeed, the degradation rate of mKO2::IκBα140-PEST(IκBαΔANK3-6) ($T_{1/2}$ = 30 min) was shorter than that of mKO2::IκBα74-PEST(IκBαΔANK1-6) (**Fig. 4c**). The longer half-life of mKO2::IκBα74-PEST(IκBαΔANK1-6) supports the hypothesis that free IκBα can accumulate in cells in which IKK is still active and, thus, can contribute to the deactivation of NFκB [37].

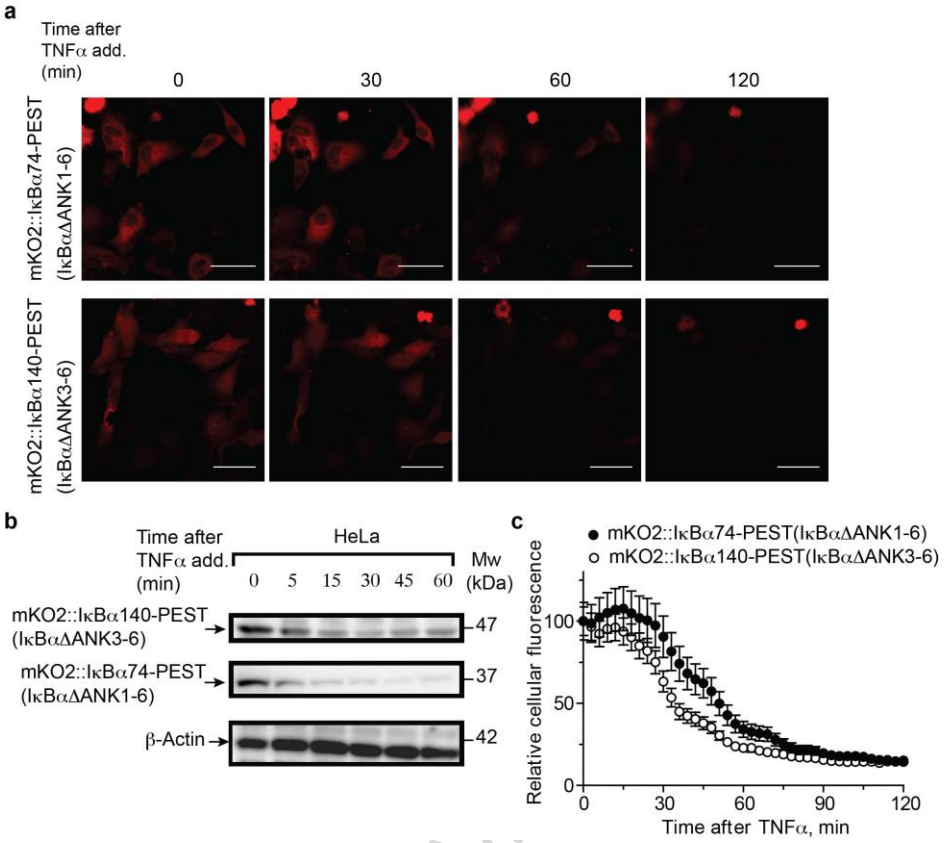

Fig. 4. **Role of the PEST sequence in TNFα-induced IκBα degradation. (a)** Time-series images of mKO2::IκBα74-PEST(IκBαΔANK1-6), and mKO2::IκBα140-PEST(IκBαΔANK3-6) fluorescence at the indicated times (min) after addition of 10 ng/mL TNFα. Scale: 50 μm. **(b)** Western blot analysis of mKO2::IκBα140-PEST(IκBαΔANK3-6) and mKO2::IκBα74-PEST(IκBαΔANK1-6) protein levels in HeLa cells stimulated with 10 ng/mL TNFα, for the indicated times. **(c)** Quantitative analysis of the time-course of IκBα degradation in HeLa cells transfected with mKO2::IκBα74-PEST(IκBαΔANK1-6) or mKO2::IκBα140-PEST(IκBαΔANK3-6) and stimulated with 10 ng/mL TNFα. The mean cellular fluorescence intensities were determined at each time-point from **(a)** and plotted as a percentage of the fluorescence at t = 0 min. Data are shown as the mean ± SD from three experiments, with at least 10 cells per experiment.

Among the fusion proteins, the degradation rate of mKO2::IκBα more closely resembled that of endogenous IκBα, suggesting that ANK repeats contribute to the degradation rate but that the PEST domain is required for the degradability of IκBα.

3.5. Effect of different proteasome inhibitors on the degradation of mKO2-IκBα fusion proteins

As the proteasome is known to degrade endogenous IκBα, we tested whether lactacystin, an irreversible and highly selective proteasome inhibitor, could block the degradation of full-length and/or mutant fusion proteins, under the tested conditions. As shown in **Fig. 5a**, we found that pretreatment of cells with 25 μM lactacystin inhibited TNFα-induced proteolysis. Time-lapse and quantitative analysis of fluorescence decay of all probes (**Fig. 5b**) indicated that mKO2::IκBα, mKO2::IκBα140-PEST(IκBαΔANK3-6), and mKO2::IκBα74-PEST(IκBαΔANK1-6) (**Supplementary movies 4, 5,** and **6**,

respectively) are degraded by the proteasome, upon TNFα stimulation.

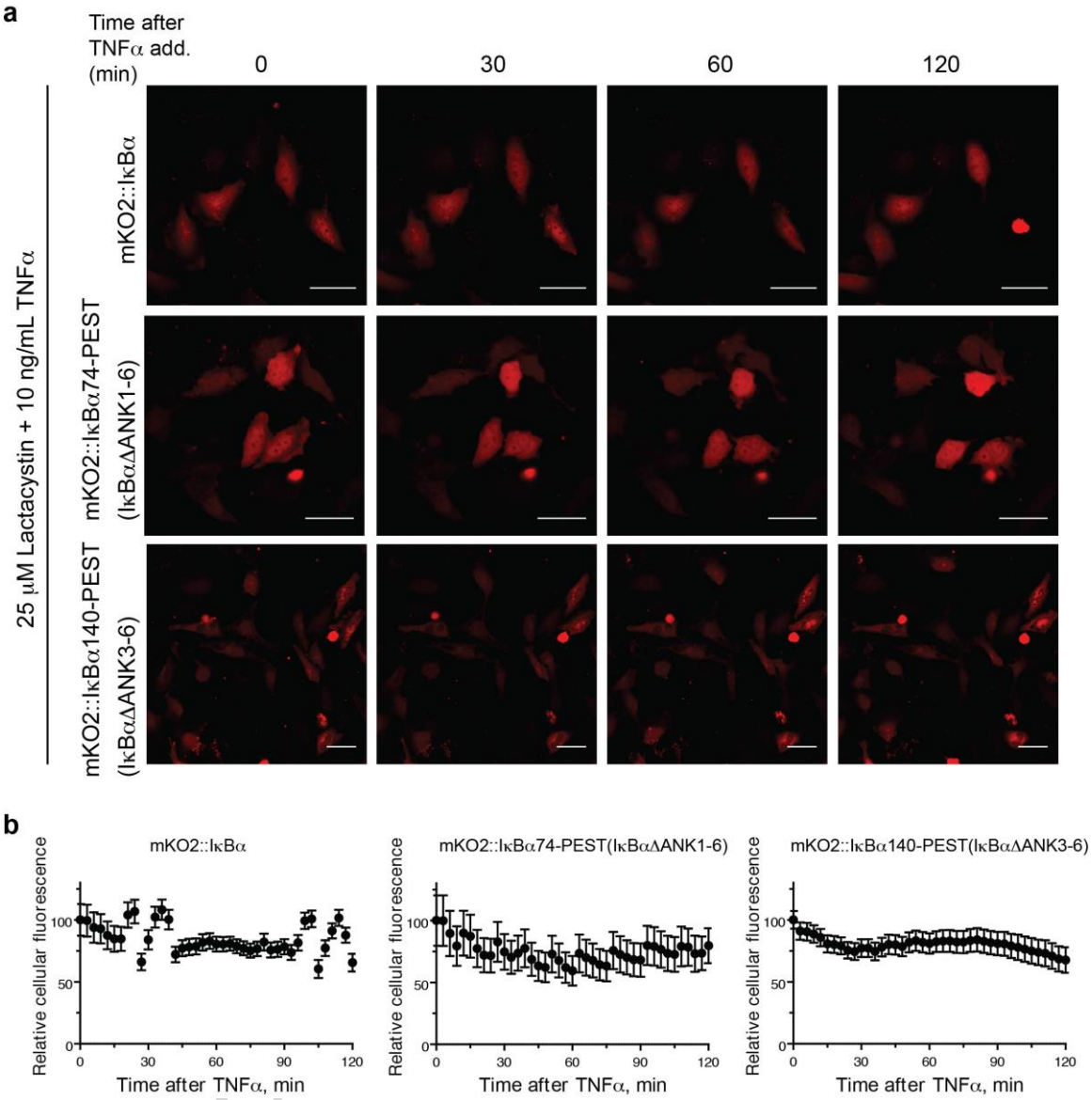

Fig. 5. Effect of lactacystin on the degradation of fusion proteins. Cells were treated with 25 μM lactacystin 24 h prior to stimulation with 10 ng/mL TNFα (t = 0 min). **(a)** Time-series images of mKO2::IκBα, mKO2::IκBα74-PEST(IκBαΔANK1-6), and mKO2::IκBα140-PEST(IκBαΔANK3-6) fluorescence at the indicated times (min) after addition of 10 ng/mL TNFα. Degradation was prevented in cells treated with 25 μM lactacystin for 24 h prior to the addition of TNFα. Scale: 50 μm. **(b)** Quantitative analysis of the time-course of IκBα degradation in HeLa cells transfected with mKO2::IκBα, mKO2::IκBα74-PEST(IκBαΔANK1-6) or mKO2::IκBα140-PEST(IκBαΔANK3-6) in response to TNFα, in the presence of lactacystin. The mean cellular fluorescence intensities were determined at each time-point and plotted as a

percentage of the fluorescence at t = 0 min. Data are shown as the mean ±SD from three experiments, with at least 10 cells per experiment.

Degradation of the aforementioned proteins was also prevented when cells were pretreated with MG132, a reversible inhibitor of proteasome activity (**Fig. 6a** and **Supplementary movies 7, 8,** and **9**, respectively).

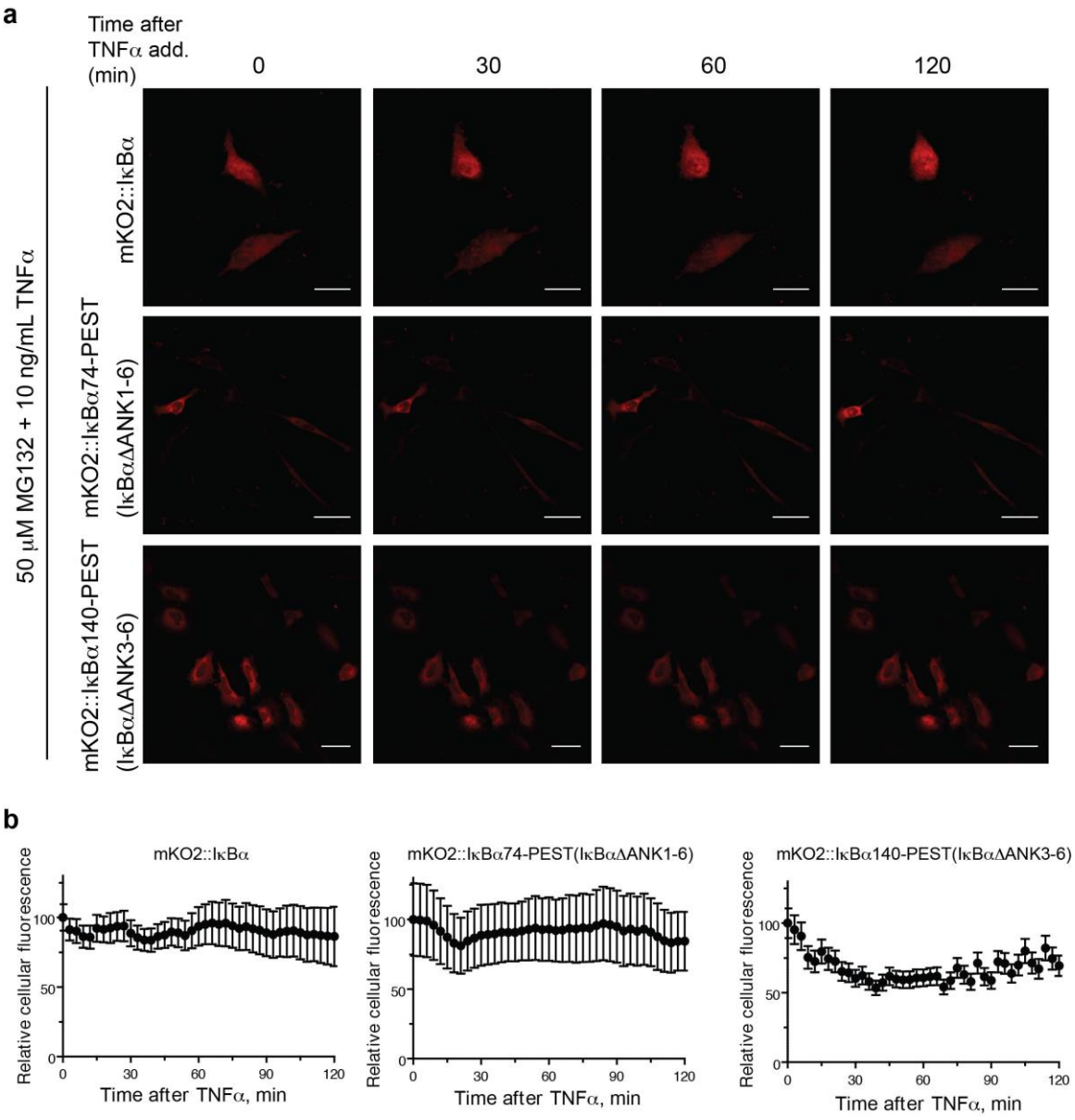

Fig. 6. Effect of MG132 on the degradation of fusion proteins. Cells were treated with 50 μM MG132, 24 h prior to stimulation with 10 ng/mL TNFα (t = 0 min). **(a)** Time-series images of mKO2::IκBα, mKO2::IκBα74-PEST(IκBαΔANK1-6), and mKO2::IκBα140-PEST(IκBαΔANK3-

6) fluorescence at the indicated times (min) after addition of 10 ng/mL TNFα. Degradation was prevented in cells treated with 50 μM MG132 for 24 h before the addition of TNFα. Scale: 50 μm. **(b)** Quantitative analysis of the time-course of IκBα degradation in HeLa cells transfected with mKO2::IκBα, mKO2::IκBα74-PEST(IκBαΔANK1-6) or mKO2::IκBα140-PEST(IκBαΔANK3-6) in response to TNFα in the presence of MG132. The mean cellular fluorescence intensities were determined at each time-point and plotted as a percentage of the fluorescence at t = 0 min. Data are shown as the mean ± SD from three experiments, with at least 10 cells per experiment.

In contrast to their different activities against the proteasome, both inhibitors have similar potencies, with K_i values of 4 nM. We used a range of concentrations of lactacystin and MG132, which were previously shown to significantly inhibit TNFα-induced proteasomal degradation of IκBα and to not induce apoptosis [39–42]. Real-time visualization allowed us to test the dose-dependent effect of lactacystin on the degradation using cells expressing mKO2::IκBα140-PEST(IκBαΔANK3-6) (**Fig. 7**), suggesting a sensitivity and applicability of this tool to screen agents targeting the proteasome pathway.

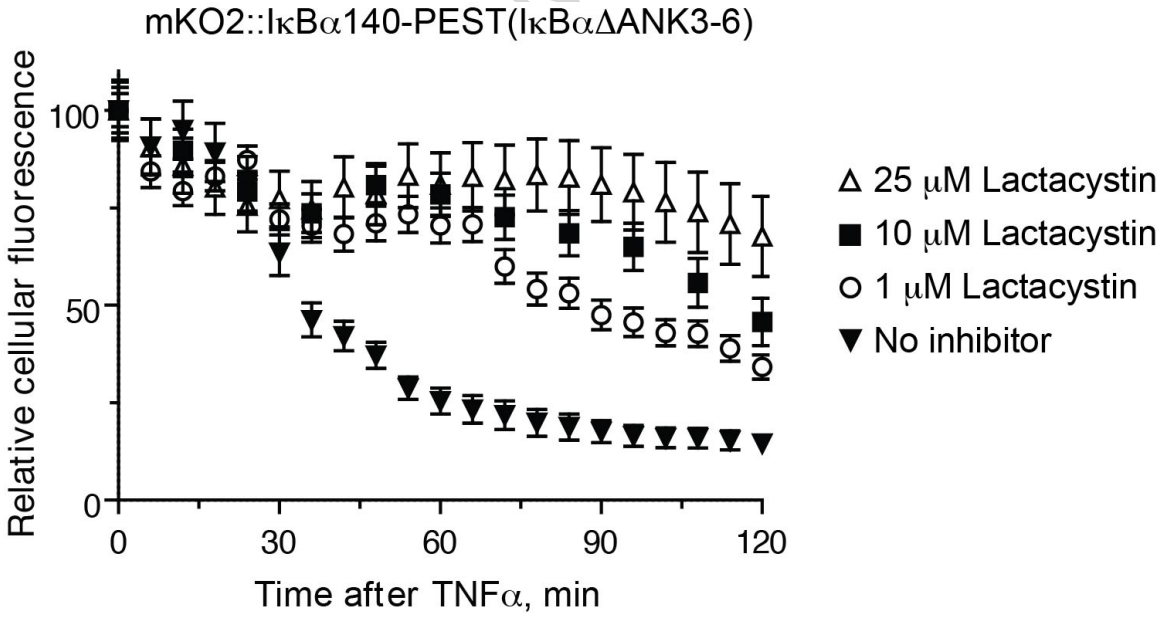

Fig. 7. Dose-dependent effect of lactacystin on the degradation of fusion proteins in response to TNFα. HeLa cells transfected with mKO2::IκBα140-PEST(IκBαΔANK3-6) were treated with 1, 10, or 25 μM lactacystin for 24 h prior to stimulation with 10 ng/mL TNFα. The mean cellular fluorescence intensities were determined at each time-point and plotted as a percentage of the

fluorescence at t = 0 min. Data are shown as the mean ± SD from three experiments, with at least 10 cells per experiment.

4. Conclusion

In this study, novel fusion proteins composed of fluorescent protein mKO2 and human IκBα were developed to evaluate the dynamics of stimulus-triggered degradation of IκBα in living cells. Real-time confocal microscopy and western blot analysis showed that, similar to endogenous IκBα, fusion-proteins expressed in HeLa cells were degraded after TNFα stimulation. Deletion studies on IκBα sequence demonstrated that the C-terminal PEST domain is essential to signal-induced degradation of IκBα and functions independently from ANK repeats. IκBα proteins without the PEST domain but containing either all six ANK repeats or solely ANK1 and 2, which are responsible for masking NF-κB NLS, failed to degrade upon TNFα stimuli. Although, ANK repeats by themselves did not affect IκBα degradability, they affected the rate of degradation. Fluorescent IκBα probes with no ANK repeats, mimicking free IκBα, degraded slower [mKO2::IκBα74-PEST(IκBαΔANK1-6); $T_{1/2}$ = 50 min] than those containing ANK 1 and 2 [mKO2::IκBα140-PEST(IκBαΔANK3-6); $T_{1/2}$ = 30 min] and the full-length IκBα (mKO2::IκBα; $T_{1/2}$ = 35 min). The degradation dynamics of ANK-containing fusion proteins were comparable to those observed with endogenous IκBα ($T_{1/2}$ = 34 min). Selective proteasome inhibitors, such as lactacystin and MG132, blocked the decay of fluorescence in a concentration-dependent manner, indicating that the fusion proteins are degraded by the proteasome system.

The technique that has been described here could have a range of possible applications, such as facilitating studies associated with IκBα dynamics and biochemical characteristics, as well as for screening of potential proteasome inhibitors.

Conflict of Interest

The authors declare no competing financial interest.

Acknowledgements

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HIGHLIGHTS

In resting cells, the nuclear factor kappa B (NF-κB) family of transcription factors is stabilized by binding with the cytoplasmic inhibitor of kappa B alpha (IκBα). We developed a novel biosensor, by fusing the monomeric fluorescent protein Kusabira-Orange 2 to IκBα (mKO2-IκBα), to study the dynamics and structure-activity relationship of IκBα degradation. The C-terminal PEST domain is required in signal-induced proteasomal degradation of IκBα. The C-terminal PEST functions independently from ankyrin repeats. IκBα ankyrin repeats do not affect IκBα degradability but affect its degradation rate. The half-life of mKO2-IκBα in response to proinflammatory stimuly is approximately 35 min, which is similar to the half-life of endogenous IκBα. Selective proteasome inhibitors, such as lactacystin and MG132, inhibit degradation and affect the kinetics of IκBα in a dose-dependent manner. The techniques described here can potentially facilitate studies associated with IκBα dynamics and biochemical characteristics, as well as the screening of potential proteasome inhibitors.