（ 続紙 1 ）

京都大学	博士（薬科学）	氏名	Nilufar Rahimova
論文題目	Real-time dynamics of IκBα degradation studied with Kusabira-Orange 2 fusion proteins (Kusabira-Orange 2 融合タンパク質による IκBα 分解のリアルタイム動態研究)		

（論文内容の要旨）

The nuclear transcription factor κB (NF-κB) controls a large number of target genes that play important roles in cell survival, inflammation and immune system, and the dysregulation of NF-κB is associated with many pathological conditions such as autoimmunity, cancers and diabetes. In resting cells, NF-κB is maintained inactive in the cytoplasm by binding to inhibitory proteins known as IκBα. Thus NF-κB control depends on the characteristics of IκBα, which has the folding dynamics that plays important roles in its functionality. Upon the pro-inflammatory stimuli provoked by such as TNFα or Toll-like receptor ligands, IκBα degrades via ubiquitin-proteasome system and leads to the activation of NF-κB, which then translocates to the nucleus and binds to its cognate gene. Thus considering potential role of IκBα, monitoring of its degradation in living cells could facilitate the understanding of dynamics of this process, as well as provide a platform for screening and evaluation of medicines targeting NF-κB signaling pathway such as proteasome inhibitors. In this study, novel genetically encoded fusion proteins composed of full-length or fragments of human IκBα (hIκBα) and fluorescent protein monomeric Kusabira-Orange 2 (mKO2) were developed to examine the involvement of different domains of IκBα in ubiquitin-proteasome degradation. Fluorescence imaging was applied to elucidate the real-time kinetics of degradation process in living cells. Furthermore, I computationally modeled these fusion proteins and performed molecular dynamic simulations in order to investigate their stability and the flexibility of the ankyrin (ANK) repeat domains in them. The refined models of fusion proteins were also used for protein-protein docking studies in order to study the structure-activity relationship.

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

Novel biosensors 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 hIκBα. Three domains of hIκBα, 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 $(GlyGlyGlyGlySer)_3$ (GS linker) in different combinations to construct mKO2::hIκBα, mKO2::hIκBα277ΔPEST, mKO2::hIκBα74ΔANK1-6PEST, mKO2::hIκBα74-PESTΔANK1-6, mKO2::hIκBα140ΔANK3-6PEST and mKO2::hIκBα140-PESTΔANK3-6. Results of western blot and sequencing PCR confirmed the expression of these six 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 tools 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

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 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 was induced by the fusion with hIκBα. Fusion proteins lacking the PEST sequence 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. Proteasome inhibitors such as lactacystin and MG132 blocked the decay of fluorescence in all PEST-containing probes, suggesting that degradation of mKO2-hIκBα fusion proteins in stimulated cells occurs via proteasomal system. The mKO2::hIκBα74-PESTΔANK1-6 and mKO2::hIκBα140-PESTΔANK3-6 proteins produced from the constructs were degraded with half-lives slightly shorter than fusion constructs with full-length IκBα, which in turn were similar to the half-life of native IκBα.

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

The three-dimensional structures of mKO2, mKO2::hIκBα, mKO2::hIκBα74-PESTΔANK1-6 and mKO2::hIκBα140-PESTΔANK3-6 were modeled using pairwise comparative (homology) modeling approach. Subsequently, molecular dynamic simulations of these proteins in the presence of explicit water were performed along with known crystal structure of hIκBα. The confidence of models (C-score) is quantified based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. Models of mKO2 (C-score=1.67, RMSD=2.3±1.8Å), mKO2::hIκBα (C-score=-0.95, RMSD=9.8±4.6Å), mKO2::hIκBα74-PESTΔANK1-6 (C-score=-0.06, RMSD=6.7±4.0Å) and mKO2::hIκBα140-PESTΔANK3-6 (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 fusion to keep distance from functional domains and N-terminal region 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 p50/p65 NF-κB subunits in order to study the effect of fusion construct to the stability of the complex. Fusion proteins did not create a single continuous buried surface contact with NF-κB but rather interacted through several independent smaller patches.

In conclusion, novel fusion proteins composed of fluorescent protein mKO2 and full-length or fragments of hIκBα were developed to evaluate dynamics of stimulus-triggered degradation of IκBα in living cells and involvement of ANK repeats and C-terminal PEST domain in degradation. Molecular-modeling approach was used to obtain a structural model and reveal the potential sites of interaction between mKO2-IκBα fusion proteins and NF-κB. Kinetics of degradation of these fusion 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 screening and evaluation of new drug candidates targeting the NF-κB signaling pathway.

（続紙 ２ ）

（論文審査の結果の要旨）

Nuclear transcription factor κB (NF-κB)は、細胞の生存、炎症・免疫反応に関わる多くの重要な遺伝子の発現を制御し、その異常は自己免疫疾患やがん・糖尿病など多くの病態に関係する。NF-κBは通常IκBαとの結合によって細胞質内で不活性化されているが、TNFαやToll様受容体リガンドの刺激により、IκBαはユビキチンープロテアソーム系を介して分解を受け、NF-κBの活性化、核内への移行と遺伝子への結合が起こる。こうしたIκBαの潜在的役割に鑑みると、生細胞におけるIκBαの分解をモニターすることはNF-κB経路の動態の把握やプロテアソーム阻害剤など医薬品の探索評価に貢献する。本研究では、IκBαのユビキチンープロテアソーム分解における各ドメインの関与を調べる目的で、IκBαないし、そのフラグメントとKusabira-Orange 2 (mKO2)蛍光タンパク質からなる新規融合タンパク質を開発し、蛍光イメージングを用いて生細胞における分解過程をリアルタイムで解析した。さらに、融合タンパク質の分子モデルを構築し分子動力学計算によって安定性やアンキリン(ANK)リピート構造のフレキシビリティを調べるとともに、タンパク質間結合における構造活性相関を検討した。

IκBαの分解のダイナミクスを追跡する新規バイオセンサーの開発にあたって、まず６つの融合タンパク質発現ベクターを構築した。これらは、hIκBαの３つのドメイン、すなわちN末リン酸化／ユビキチン化ドメイン、ANKリピート1、2ドメイン、C末PESTドメインを適宜組み合わせ $(GlyGlyGlyGlySer)_3$リンカーを介してmKO2と結合したものである。PCR配列解析およびウェスタンブロットで目的遺伝子・タンパク質の発現を確認後、HeLa細胞にトランスフェクションしてmKO2の蛍光シグナルが得られることも確認した。

次に、リアルタイム共焦点顕微鏡法およびウェスタンブロット法を用い、HeLa細胞で発現した融合タンパク質がTNFα刺激で分解することを確認した。融合タンパク質の蛍光強度の減少はTNFα刺激後約7分で観察され、内在性IκBαの分解と同様であった。一方、mKO2単独では蛍光減少が起こらないことから、その分解はhIκBαとの融合によることが示された。また、PEST配列を含まないあるいはその配列に変異のある融合タンパク質では刺激による分解誘導は起こらず、PEST配列はIκBαの分解に必須であることが結論された。さらに、プロテアソーム阻害剤のlactacystin およびMG132が蛍光の減少を抑制したことから、mKO2-hIκBα融合タンパク質の分解がプロテアソーム系を介することも確認できた。ANKリピート配列を完全にあるいはANK3-6の部分を欠失した融合タンパク質では完全長hIκBαの融合タンパク質に比べ分解半減期が短く、結果として本来のIκBαと同程度であった。

mKO2および３つの融合タンパク質mKO2::hIκBα、mKO2::hIκBα74-PESTΔANK1-6、mKO2::hIκBα140-PESTΔANK3-6について、ホモロジーモデリング法により立体構造を推定した後、水分子を顕に考慮した条件下で分子動力学シミュレーションを行い、ANKリピートドメインのフレキシビリティおよび複合体安定性に対する融合体構造の

影響を解析した。スレッドアラインメントと構造アセンブリシミュレーションの収束から定式化される信頼度スコアに基づく評価から、各立体構造予測モデルは適切なグローバルトポロジーを有していた。さらに得られたモデルでp65とのタンパク質間ドッキングを行い、NF-κB活性化および阻害の構造基盤を明らかにした。

以上、申請者は蛍光タンパク質mKO2と完全長hIκBαあるいはフラグメントとの融合タンパク質を開発することによって、生細胞における刺激応答IκBα分解のダイナミクスおよび分解におけるANKリピートやC末PESTドメインの関与を評価した。プロテアソーム阻害剤での検討により、炎症誘発性刺激下での融合タンパク質の分解キネティクスをリアルタイムモニタリングする方法が、NF-κBシグナル経路を標的とする薬物候補のスクリーニングや評価のためのプラットフォームとなることを例証した。

よって、本論文は博士（薬学）の学位論文として価値あるものと認める。また、平成２８年８月２５日、論文内容とそれに関連した事項について諮問を行った結果、合格と認めた。

なお、本論文は、京都大学学位規程第１４条第２項に該当するものと判断し、公表に際しては、（当分の間）当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降