Regulation of mIκBNS stability through PEST-mediated degradation by proteasome

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ABSTRACT

Negative regulatory proteins in a cytokine signaling play a critical role in restricting unwanted excess activation of the signaling pathway. At the same time, negative regulatory proteins need to be removed rapidly from cells to respond properly to the next incoming signal. A nuclear IκB protein called IκBNS is known to inhibit a subset of NF-κB target genes upon its expression by NF-κB activation. Here, we show a mechanism to control the stability of mIκBNS which might be important for cells to prepare the next round signaling. We found that mIκBNS is a short-lived protein of which the stability is controlled by proteasome, independent of ubiquitylation process. We identified that the N-terminal PEST sequence in mIκBNS was critical for the regulation of stability.

1. Introduction

Cytokine signaling is crucial for a variety of physiological responses in the body. The intensity and duration of the signaling must be tightly regulated since unlimited activation of cytokine signaling is harmful or sometimes detrimental to the body. Many tiers of negative regulatory mechanisms to restrict such an over activation of cytokine response has been well studied, including endocytosis and degradation of ligand-bound receptors, action of protein phosphatases, and negative feedback inhibition by newly synthesized proteins as a result of the cytokine signaling [1,2]. Interestingly, it has shown that knockout of several negative regulators in the cytokine signaling could cause lethal shock in mice because of unlimited activation of the signaling [3–6], suggesting the physiological significance of the tight regulation of cytokine signaling through several layers of regulation mechanisms.

One interesting nature of cytokine-induced negative regulators such as suppressor of cytokine signaling 1 (SOCS1), SOCS3, UBP43, and IκBα is their short half-lives, which are regulated by ubiquitin–proteasome system or by proteasome only [7–10]. SOCS-1 was stabilized by proteasome inhibitor and TRIM8 has suggested as an ubiquitin E3 ligase for SOCS-1 [8,11]. The stability of SOCS-3 is regulated mainly through ubiquitylation on Lys-6 [12]. In addition, unstructured PEST sequence in the N-terminal SH2

domain of SOCS-3 has been proposed to be involved in the regulation of the stability [13,14]. UBP43, a negative regulator specific for interferon-α/β signaling, has been shown to be expressed in the bone marrow-derived macrophages upon lipopolysaccharide (LPS) treatment and rapidly removed from the cells in a relatively short period of time [5], and the SCF^{skp2} ubiquitin E3 ligase complex was suggested to control the stability of UBP43 [9]. The control mechanism of IκBα stability has been well established. NF-κB-bound IκBα is phosphorylated by activated IκB kinase upon cytokine signaling which led ubiquitin-dependent degradation of IκBα [15]. On the other hand, the stability of free IκBα is controlled intrinsically by the PEST sequence in the C-terminal region of IκBα rather than by phosphorylation-mediated ubiquitylation [10].

IκBNS belongs to nuclear IκB protein which was originally identified as an inducible gene upon activation of T cell receptors in thymocytes [16]. Knockout of IκBNS caused defect in proliferation and function of T cells and B cells [17,18]. Especially, IκBNS mediates Foxp3 expression in regulatory T (Treg) cells and contributes to the maturation of Treg precursor cells into Foxp3(+) cells [19]. On the contrary to its positive role for gene expression in adaptive immune system, IκBNS is likely to function as a negative regulator for a certain gene expression in innate immune system. IκBNS-deficiency caused hyper-expression of a subset of genes (secondary phase genes among NF-κB target genes) including IL-6 and IL-12 p40 upon LPS-administration to MEFs and macrophages, suggesting its negative regulatory role on certain NF-κB target genes [20].

We report that mIκBNS is an unstable protein which is degraded by proteasome. We found putative PEST sequence at the N-terminal region of mIκBNS and elucidated that this PEST sequence is a critical determinant for the rapid degradation of

mIκBNS. We also identified a nuclear localization signal at the C-terminal region of mIκBNS supporting that the longer half-life of PEST mutant mIκBNS is not because of the mislocalization of the protein. Taken together, PEST-mediated regulation of mIκBNS stability might be important for rapid response to the next round signaling.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Full-length cDNA for mouse IκBNS (mIκBNS) was generated by RT-PCR using mRNA purified from LPS-treated bone marrow-derived macrophages as a template and cloned into p3xFlagCMV10 vector (Sigma–Aldrich, USA) using HindIII and BglII digestion. Mutagenesis for the replacement of lysine residues to arginine (K98R/K100R, K153R, K219R/K222R, K255R, and K316R) in mIκBNS was performed using QuickChange XL site-directed mutagenesis kit (Stratagene, USA) according to manufacturer's instructions. Mutated constructs were confirmed by sequencing and subcloned into p3xFlagCMV10 vector. To generate PESTA mutant form of mIκBNS in which serine and threonine residues in PEST sequence were replaced to alanine as indicated in Fig. 3, the corresponding mutant nucleotide sequence was synthesized by GenScript (USA) and cloned into p3xFlagCMV10 vector. Nuclear localization sequence (NLS) mutant form of mIκBNS was generated by PCR using 3′ reverse primer containing mutations.

2.2. Cell culture and transfections

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen, USA). Cultured cells were transfected with plasmid DNA using PolyFect reagent (Quiagen, Germany) according to the manufacturer's protocol. The cells were harvested for subsequent applications at indicated time periods after transfection.

2.3. Western blot analysis and antibodies

Protein extracts were prepared in RIPA buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40). Twenty micrograms of protein extracts were run on SDS–PAGE using a 8–15% gradient gel and transferred to Hybond ECL nitrocellulose membrane (GE Healthcare, UK). After blocking with 3% non-fat milk, the membrane was incubated with the appropriate antibodies, followed by incubation with secondary antibodies. Tubulin-α was used as a marker of equal loading. The proteins were visualized with Pico EPD Western Blot Detection Kit (ELPIS Biotech, South Korea) and analyzed by LAS-4000 luminescent image analyzer (Fuzi Film, Japan). Antibodies were purchased from the manufacturers as follows; anti-Flag and anti-tubulin-α (Sigma–Aldrich, USA), and anti-Lamin A/C (Santa Cruz, USA).

2.4. Protein stability assay

HeLa cells plated on 100 mm dishes were transfected with plasmids expressing wild-type or mutant forms of mIκBNS. After 24 h of transfection, cells were divided onto 6-well plates. On the next day, cells were treated with cycloheximide (CHX) to be 20 μg/ml in total. Cells were harvested at the indicated periods of time after CHX treatment and applied for immunoblotting with anti-Flag antibody. MG132, a proteasome inhibitor, was added together with CHX to be 10 μM in total (Sigma–Aldrich, USA). Control cells were treated with dimethyl sulphoxide (DMSO).

2.5. Preparation of nuclear extract

HeLa cells were cultured on 60 mm dishes and transfected with plasmids expressing wild-type or mutant forms of mIκBNS. After 36 h of transfection, cells were washed twice with ice cold PBS and harvested. Cells were resuspended in 400 μl of hypotonic buffer (25 mM Tris–HCl, pH 8.0, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), incubated on ice for 15 min for swelling, and then 25 μl of 10% NP-40 was added to the cells. After centrifugation at 15,000g for 2 min at 4 °C, supernatants were collected as cytoplasmic extracts. Pellets were resuspended in ice cold high-salt buffer (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 1 mM DTT, 1 mM PMSF) and incubated on a shacking platform for 10 min at 4 °C. After centrifugation at 20,000g for 5 min, the supernatant was collected as nuclear extracts.

3. Results

3.1. mIκBNS stability is controlled by proteasome

Based on the short half-lives of IκB proteins, we questioned whether IκBNS is also an unstable protein in cells. We used mouse IκBNS sequence which are tagged with 3 copies of Flag epitopes (Flag-mIκBNS) for stability assay. Flag-mIκBNS was transfected into HeLa cells and the stability of the protein was measured after inhibition of new protein synthesis by treating cells with cycloheximide (CHX) and then chasing the level of cellular Flag-mIκBNS. Cellular level of Flag-mIκBNS decreased significantly over time in the presence of CHX, while the level was consistent without CHX treatment (Fig. 1A and C), indicating the unstable nature of Flag-mIκBNS protein in the cells. In addition, the half-life of
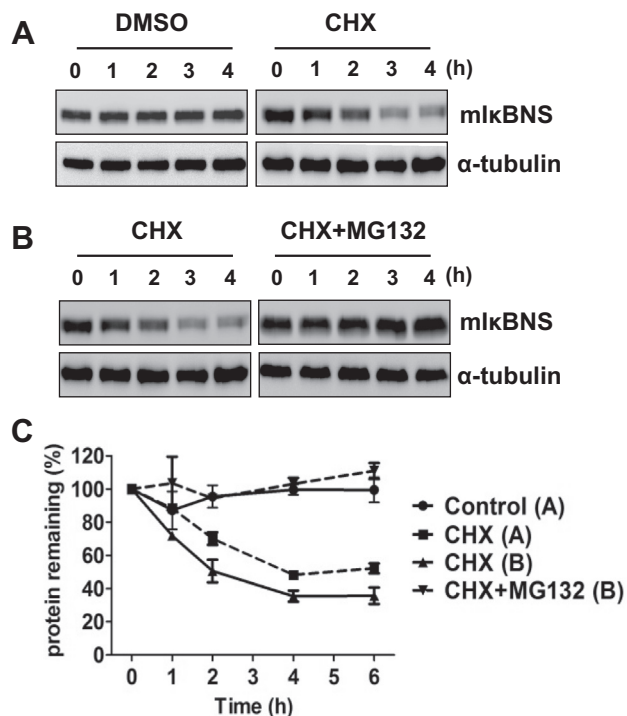
Fig. 1. Proteasomal degradation of mIκBNS. (A) HeLa cells expressing Flag-mIκBNS were treated with either DMSO or cycloheximide (CHX) for indicated time periods. Cells were harvested and analyzed by immunoblotting using anti-Flag antibody. (B) HeLa cells expressing Flag-mIκBNS were treated with either CHX alone or with CHX and proteasome inhibitor, MG132 (10 μM in total). Flag-mIκBNS was detected as described in (A). (C) Triplicate experiments in (A) and (B) are represented graphically with error bars.