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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

Abbreviations: SOCS, suppressor of cytokine signaling; LPS, lipopolysaccharide; NLS, nuclear localization sequence; CHX, cycloheximide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

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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.

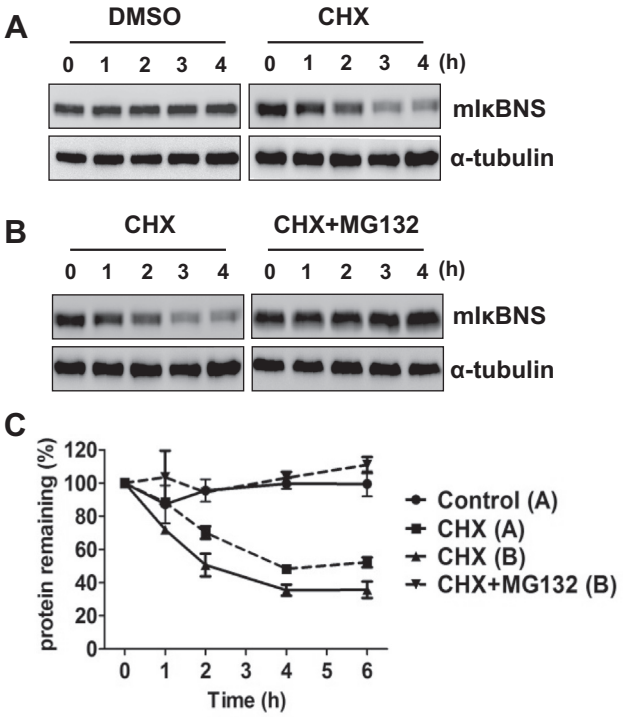

Fig. 1. Proteasomal degradation of mIκBNS. (A) HeLa cells expressing Flag-mIκBNS were treated with either DMSO or cycloheximide (CHX) for indicated time periods. Cells were harvested and analyzed by immunoblotting using anti-Flag antibody. (B) HeLa cells expressing Flag-mIκBNS were treated with either 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.

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 15,000*g* 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 20,000*g* 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

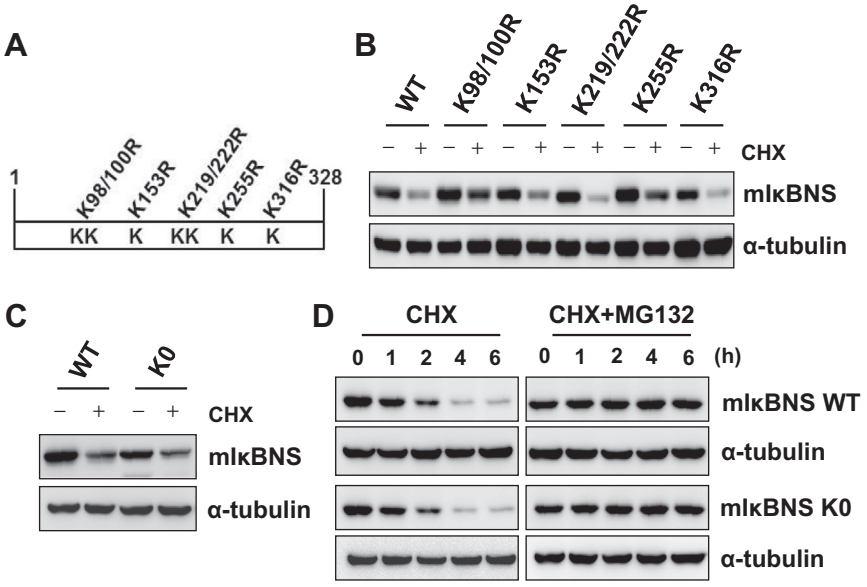

Fig. 2. No involvement of ubiquitylation in mIκBNS degradation. (A) Schematic representation of 7 lysine residues in mIκBNS and its mutation to arginine residues. (B) HeLa cells expressing wild-type or each mutant form of Flag-mIκBNS were treated with either DMSO or CHX for 6 h. Flag-mIκBNS proteins were detected by immunoblotting against Flag epitope. (C) The stability of two forms of mIκBNS, wild-type and K0 mutant in which all 7 lysine residues were substituted to arginine residues, was measured as described in (B). (D) The stability of wild-type and K0 mutant form of mIκBNS was compared in the presence or absence of MG132.

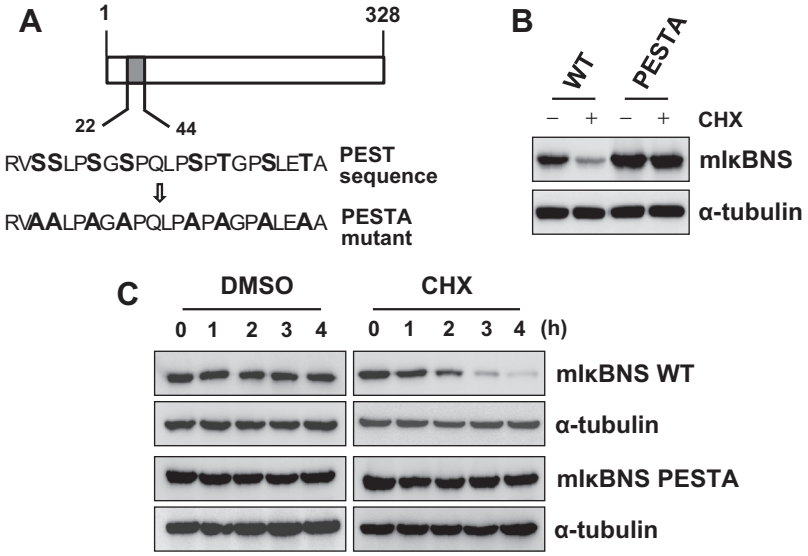

Fig. 3. Identification of PEST sequence which is responsible for the unstable nature of mIκBNS. (A) Schematic representation of PEST sequence at the N-terminal region of mIκBNS and PESTA mutant in which 6 serine and 2 threonine residues in PEST sequence were replaced to alanines. (B) The stability of wild-type and PESTA mutant form of mIκBNS was compared after treating cells with either DMSO or CHX for 6 h as described in Fig. 2B. (C) The stability of wild-type and PESTA mutant form of mIκBNS was compared in time course after treating cells with CHX.

Flag-mIκBNS increased greatly in the presence of proteasome inhibitor, MG132 (Fig. 1B and C). Taken together, these results suggested that proteasomal degradation is responsible for the unstable nature of mIκBNS.

3.2. Proteasome but no ubiquitylation is involved in the regulation of mIκBNS stability

We examined whether ubiquitylation of Flag-mIκBNS is necessary for the proteasomal degradation of the protein. However, we did not obtain any obvious evidence for the ubiquitylation of Flag-mIκBNS in transfection-based ubiquitylation assays (data not shown). We then tested whether the substitution of lysine residues in mIκBNS to arginine can enhance the stability of the protein. As we showed in Fig. 2A, mIκBNS contains 7 lysine residues. We generated five mutant forms of mIκBNS by substituting each lysine residue to arginine or exchanging two closely located lysine residues to arginine, and compared the stability of wild-type protein with each mutant protein in the presence of CHX (Fig. 2B). None of the mutant mIκBNS proteins exhibited significant increase in its stability compared to wild-type protein (Fig. 2B). In addition, K0 mutant form of mIκBNS in which all seven lysine residues were substituted to arginine still showed similar half-life with wild-type protein (Fig. 2C), suggesting that ubiquitylation of mIκBNS is not a prerequisite for the degradation of the protein. Furthermore, the stability of mIκBNS-K0 is enhanced in the presence of MG132

(Fig. 2D). Taken together, mIκBNS is an unstable protein with short half-life in cells, which is regulated by proteasome but independent of ubiquitylation.

3.3. N-terminal PEST region of mIκBNS is critical for its unstable nature

As we identified unstable nature of mIκBNS protein with no sign of ubiquitylation, we searched for the possible cause of short half-life of mIκBNS. One strong candidate mechanism that may control mIκBNS stability is intrinsic instability of mIκBNS, caused by the PEST sequence as others observed in free IκBα [10]. We analyzed amino acid sequence of mIκBNS using PESTfind analysis webtool (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). As shown in Fig. 3A, we found potential PEST sequence at the N-terminal region of mIκBNS with score of +5.39. In order to address whether this sequence governs the unstable nature of mIκBNS, we generated mutant protein called mIκBNS-PESTA by replacing 6 serine and 2 threonine residues in putative PEST region to alanines (Fig. 3A), as others did to validate PEST sequence in IκBα [10]. We then compared the stability of transiently expressed wild-type and PESTA forms of mIκBNS in HeLa cells in the presence of CHX. Interestingly, the half-life of Flag-mIκBNS-PESTA increased greatly compared to wild-type protein (Fig. 3B and C), indicating that the PEST region is indeed critical for the stability control of mIκBNS. Thus we concluded that the N-terminal PEST sequence in mIκBNS is critical for its intrinsic unstable nature and a determinant for proteasomal degradation of the protein.

3.4. C-terminal nuclear localization signal but no PEST region is determinant for nuclear localization of mIκBNS

One concern was that the stabilization of mIκBNS-PESTA mutant might be caused by the defect in proper nuclear localization of mIκBNS rather than increased intrinsic stability against proteasomal degradation, especially because the nuclear localization sequence (NLS) of IκBNS was proposed to be located in the N-terminal region of the protein [21]. However, there was no report pinpointing the NLS sequence of IκBNS and proving it experimentally. We searched for the potential NLS in mIκBNS using web-based program called cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), and found a single putative NLS sequence at the C-terminus of mIκBNS (Fig. 4A). To test whether the sequence is necessary for the nuclear localization of mIκBNS, we substituted three basic amino acid residues, 316th lysine, 317th arginine, and 319th arginine to alanine (Fig. 4A). We then examined the cellular localization of Flag-tagged wild-type, PESTA mutant, and NLS mutant form (mIκBNS-NLSA) of mIκBNS proteins which were transiently expressed in HeLa cells. Nuclear and cytoplasmic fractions from each transfected cells were analyzed by immunoblotting against Flag-tag and the purity of each fraction was validated by showing exclusive signal of Lamin A/C or Tubulin-α for nuclear or cytoplasmic fraction, respectively. Wild-type and PESTA mutant forms of mIκBNS protein were detected in both nuclear and cytoplasmic fractions, while most of mIκBNS-NLSA protein was predominantly located in cytoplasm (Fig. 4B), indicating that the putative NLS at the C-terminus of mIκBNS is bona fide nuclear localization signal. Interestingly, when we compared the stability of wild-type, PESTA, and NLSA forms of mIκBNS, NLSA showed similar short half-life with wild-type protein (Fig. 4C), implying that NLS region is not critical for proteasomal degradation of mIκBNS. These results confirm that the N-terminal PEST sequence is the critical determinant for unstable nature of mIκBNS.

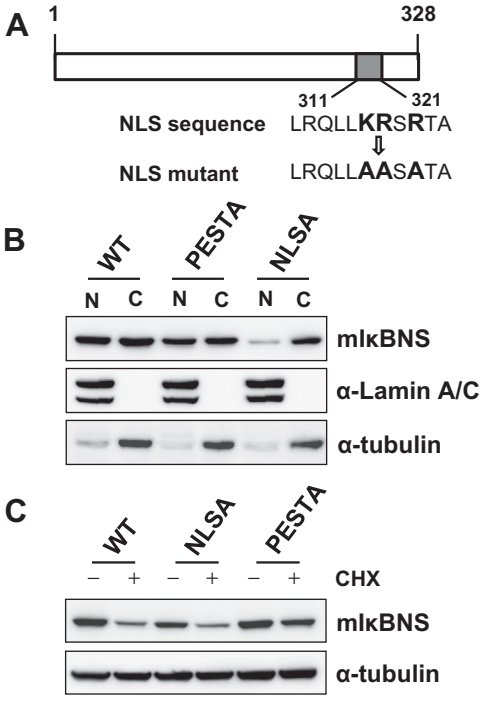

Fig. 4. Nuclear localization signal (NLS) is not critical for mIκBNS degradation. (A) Schematic representation of NLS sequence at the C-terminal region of mIκBNS and NLS mutant (NLSA) in which one lysine and two arginine residues were replaced to alanines. (B) Nuclear or cytoplasmic localization of wild-type, PESTA, or NLSA forms of mIκBNS was determined by cellular fractionation followed by immunoblotting against Flag epitope. The quality of fractions was validated by immunoblotting against Lamin A/C for nuclear fraction and tubulin-α for cytoplasmic fraction. (C) The stability of wild-type, NLSA, and PESTA mutant form of mIκBNS was compared after treating cells with either DMSO or CHX for 6 h as described in Fig. 2B.

4. Discussion

Regulation of protein stability is critical to many cellular processes such as cell cycle and cytokine signaling [1,2,22,23]. In cytokine signaling, the expression of negative regulators is often induced by the activation of the same signaling pathway, so that the regulator can in turn contribute to the termination of the signaling [1,2]. A general nature of these cytokine-induced negative regulators is that they have short half-lives. IκBNS is known to negatively regulate a subset of NF-κB target genes in response to inflammatory signaling [20]. However, there is no report about the regulatory mechanism of IκBNS stability.

We questioned whether IκBNS has short half-life and, if so, what molecular determinant governs the unstable characteristics of IκBNS. We found that mouse form of IκBNS was rapidly degraded in cells by proteasome. Ubiquitylation-mediated degradation was a strong candidate mechanism for the degradation of mIκBNS; however, ubiquitylation was not critical based on the following evidence. First, we did not detect any significant ubiquitylation of mIκBNS, even in the presence of proteasome inhibitor. Second, substitution of all 7 lysine residues to alanine in mIκBNS failed to stabilize mIκBNS. Although ubiquitylation on the N-terminal amino acid residue has been reported [24], we ruled out the possibility because various tagging on the N-terminus of mIκBNS did not change the stability (data not shown). To search the intrinsic molecular determinant for the unstable nature of mIκBNS, we found PEST sequence, which is well-known amino acid sequences commonly found in rapidly degraded proteins [25], at the N-terminal region of mIκBNS with score of +5.39. This score is comparable to the score of C-terminal PEST sequences in human IκBα (+5.13) and mouse IκBα (+4.59), which are calculated

using the same PESTfind program. On the contrary to high PEST scores in both human and mouse IκBα, human form of IκBNS did not contain significant PEST sequences. Thus, unstable nature of mIκBNS governed by PEST sequence might be species specific. IκBα stability is regulated by dual mechanism depending on its binding status with NF-κB. NF-κB-bound IκBα is degraded by proteasome through phosphorylation-mediated ubiquitylation [26]. On the other hand, the stability control mechanism of free IκBα is quite similar to that of mIκBNS. Both proteins are intrinsically unstable and degraded by proteasome without ubiquitylation [10]. In addition, PEST sequences are responsible for the rapid degradation of both proteins. Since IκBNS plays a role only in the negative regulation of innate immune signaling without involving in the activation of the signaling, the mechanism to control IκBNS stability seems to be relatively simple.

IκBNS is a nuclear protein but its NLS is not defined clearly. Since the NLS of IκBNS was proposed to be located in the N-terminal region of the protein [21], we had to rule out the possibility that mutation of PEST sequences might affect nuclear localization of the protein. Since the percentage of nuclear fraction was the same between wild-type and PEST mutant form of mIκBNS, we concluded that PEST sequence is not involved in the nuclear localization of mIκBNS. In the meantime, we found single putative NLS located at the C-terminus of mIκBNS and confirmed experimentally its critical role for nuclear localization of the protein. This NLS sequence is very well-conserved between mouse and human IκBNS. In our study, we elucidated the stability control mechanism for mIκBNS, which is governed by PEST-mediated proteasomal degradation. We believe that this mechanism is critical for the regulation of responsiveness to cytokine signaling.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] A. Yoshimura, Negative regulation of cytokine signaling, Clin. Rev. Allergy Immunol. 28 (2005) 205–220.
[2] T. Lang, A. Mansell, The negative regulation of Toll-like receptor and associated pathways, Immunol. Cell Biol. 85 (2007) 425–434.
[3] B. Liu, S. Mink, K.A. Wong, N. Stein, C. Getman, P.W. Dempsey, H. Wu, K. Shuai, PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity, Nat. Immunol. 5 (2004) 891–898.
[4] R. Nakagawa, T. Naka, H. Tsutsui, M. Fujimoto, A. Kimura, T. Abe, E. Seki, S. Sato, O. Takeuchi, K. Takeda, S. Akira, K. Yamanishi, I. Kawase, K. Nakanishi, T. Kishimoto, SOCS-1 participates in negative regulation of LPS responses, Immunity 17 (2002) 677–687.
[5] K.I. Kim, O.A. Malakhova, K. Hoebe, M. Yan, B. Beutler, D.E. Zhang, Enhanced antibacterial potential in *UBP43*-deficient mice against *Salmonella typhimurium* infection by up-regulating type I IFN signaling, J. Immunol. 175 (2005) 847–854.
[6] O.A. Malakhova, M. Yan, M.P. Malakhov, Y. Yuan, K.J. Ritchie, K.I. Kim, L.F. Peterson, K. Shuai, D.E. Zhang, Protein ISGylation modulates the JAK-STAT signaling pathway, Genes Dev. 17 (2003) 455–460.
[7] J.G. Zhang, A. Farley, S.E. Nicholson, T.A. Willson, L.M. Zugaro, R.J. Simpson, R.L. Moritz, D. Cary, R. Richardson, G. Hausmann, B.J. Kile, S.B. Kent, W.S. Alexander, D. Metcalf, D.J. Hilton, N.A. Nicola, M. Baca, The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 2071–2076.
[8] T. Kamura, S. Sato, D. Haque, L. Liu, W.G. Kaelin Jr., R.C. Conaway, J.W. Conaway, The elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families, Genes Dev. 12 (1998) 3872–3881.
[9] S. Tokarz, C. Berset, J. La Rue, K. Friedman, K. Nakayama, K. Nakayama, D.E. Zhang, S. Lanker, The ISG15 isopeptidase UBP43 is regulated by proteolysis via the SCF^{Skp2} ubiquitin ligase, J. Biol. Chem. 279 (2004) 46424–46430.
[10] E. Mathes, E.L. O'Dea, A. Hoffmann, G. Ghosh, NF-κB dictates the degradation pathway of IκBα, EMBO J. 27 (2008) 1357–1367.
[11] E. Toniato, X.P. Chen, J. Losman, V. Flati, L. Donahue, P. Rothman, TRIM8/GERP RING finger protein interacts with SOCS-1, J. Biol. Chem. 277 (2002) 37315–37322.
[12] A. Sasaki, K. Inagaki-Ohara, T. Yoshida, A. Yamanaka, M. Sasaki, H. Yasukawa, A.E. Koromilas, A. Yoshimura, The N-terminal truncated isoform of SOCS3 translated from an alternative initiation AUG codon under stress conditions is stable due to the lack of a major ubiquitination site, Lys-6, J. Biol. Chem. 278 (2003) 2432–2436.
[13] J.J. Babon, S. Yao, D.P. DeSouza, C.F. Harrison, L.J. Fabri, E. Liepinsh, S.D. Scrofani, M. Baca, R.S. Norton, Secondary structure assignment of mouse SOCS3 by NMR defines the domain boundaries and identifies an unstructured insertion in the SH2 domain, FEBS J. 272 (2005) 6120–6130.
[14] J.J. Babon, E.J. McManus, S. Yao, D.P. DeSouza, L.A. Mielke, N.S. Sprigg, T.A. Willson, D.J. Hilton, N.A. Nicola, M. Baca, S.E. Nicholson, R.S. Norton, The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability, Mol. Cell 22 (2006) 205–216.
[15] M. Karin, Y. Ben-Neriah, Phosphorylation meets ubiquitination: the control of NF-κB activity, Annu. Rev. Immunol. 18 (2000) 621–663.
[16] E. Fiorini, I. Schmitz, W.E. Marissen, S.L. Osborn, M. Touma, T. Sasada, P.A. Reche, E.V. Tibaldi, R.E. Hussey, A.M. Kruisbeek, E.L. Reinherz, L.K. Clayton, Peptide-induced negative selection of thymocytes activates transcription of an NF-κB inhibitor, Mol. Cell 9 (2002) 637–648.
[17] M. Touma, V. Antonini, M. Kumar, S.L. Osborn, A.M. Bobenchik, D.B. Keskin, J.E. Connolly, M.J. Grusby, E.L. Reinherz, L.K. Clayton, Functional role for IκBNS in T cell cytokine regulation as revealed by targeted gene disruption, J. Immunol. 179 (2007) 1681–1692.
[18] M. Touma, D.B. Keskin, F. Shiroki, I. Saito, S. Koyasu, E.L. Reinherz, L.K. Clayton, Impaired B cell development and function in the absence of IκBNS, J. Immunol. 187 (2011) 3942–3952.
[19] M. Schuster, R. Glauben, C. Plaza-Sirvent, L. Schreiber, M. Annemann, S. Floess, A.A. Kuhl, L.K. Clayton, T. Sparwasser, K. Schulze-Osthoff, K. Pfeffer, J. Huehn, B. Siegmund, I. Schmitz, IκBNS protein mediates regulatory T cell development via induction of the Foxp3 transcription factor, Immunity 37 (2012) 998–1008.
[20] H. Kuwata, M. Matsumoto, K. Atarashi, H. Morishita, T. Hirotani, R. Koga, K. Takeda, IκBNS inhibits induction of a subset of Toll-like receptor-dependent genes and limits inflammation, Immunity 24 (2006) 41–51.
[21] M. Yamamoto, K. Takeda, Role of nuclear IκB proteins in the regulation of host immune responses, J. Infect. Chemother. 14 (2008) 265–269.
[22] L.K. Teixeira, S.I. Reed, Ubiquitin ligases and cell cycle control, Annu. Rev. Biochem. 82 (2013) 387–414.
[23] I. Sumara, S. Maerki, M. Peter, E3 ubiquitin ligases and mitosis: embracing the complexity, Trends Cell Biol. 18 (2008) 84–94.
[24] A. Ciechanover, R. Ben-Saadon, N-terminal ubiquitination: more protein substrates join in, Trends Cell Biol. 14 (2004) 103–106.
[25] S. Rogers, R. Wells, M. Rechsteiner, Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, Science 234 (1986) 364–368.
[26] E. Zandi, Y. Chen, M. Karin, Direct phosphorylation of IκB by IKKα and IKKβ: discrimination between free and NF-κB-bound substrate, Science 281 (1998) 1360–1363.