CSN3 interacts with IKKγ and inhibits TNF- but not IL-1-induced NF-κB activation

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Abstract The transcription factor nuclear factor κB (NF-κB) plays a pivotal role in immune and inflammatory responses. Activation of NF-κB requires the activity of IKK, a kinase complex that contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ. To understand how IKK activity is regulated, we searched for IKKγ-interacting proteins by the yeast two-hybrid system. These screenings identified CSN3, a component of the COP9 signalsome, as a protein specifically interacting with IKKγ. Overexpression of CSN3 inhibits NF-κB activation triggered by tumor necrosis factor (TNF), but not interleukin-1 (IL-1). Moreover, overexpression of CSN3 also inhibits NF-κB activation triggered by proteins involved in TNF signaling, including TNF-R1, TRAF2, RIP, and NIK, but not by TRAF6, a protein involved in IL-1 signaling. These data suggest that CSN3 is a specific negative regulator of TNF- but not IL-1-induced NF-κB activation pathways. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Two-hybrid; Nuclear factor κB; Signaling; CSN3; IKKγ

1. Introduction

Nuclear factor-κB (NF-κB) proteins are inducible transcription factors controlling the expression of various genes involved in inflammatory and immune responses [1–4]. Under physiological conditions, NF-κB is normally sequestered in the cytoplasm through its association with inhibitors called IκB [1–4]. In response to various stimuli, such as the proinflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1), IκBs are degraded and NF-κB is released to enter the nucleus [1–4]. The proteasome-mediated degradation of IκBs is initiated by phosphorylation on their specific serine residues [5,6]. The kinase responsible for the site-specific phosphorylation of IκBs is called IKK, a 900 kDa protein complex consisting of IKKα, IKKβ, and IKKγ/NEMO [7–13]. In this complex, IKKα and IKKβ are two homologous catalytic subunits which can directly phosphorylate IκBs, while IKKγ is a non-enzymatic regulatory subunit [7–13].

IKKγ was first identified in a genetic screening experiment for components required for lipopolysaccharide (LPS)-induced NF-κB activation [13]. It was soon found that IKKγ is a component of the IKK complex [11]. IKKγ-deficient cell lines are unable to respond to NF-κB-activating signals, including TNF, IL-1 and LPS, suggesting that IKKγ is required for NF-κB activation triggered by these divergent stimuli [11–14]. IKKγ contains one leucine-zipper motif at its C-terminus and two coiled-coil regions at its N-terminus and intermediate region [11]. A mutant lacking the C-terminal leucine-zipper motif can inhibit TNF-induced NF-κB activation, suggesting that the C-terminus of IKKγ is critically involved in NF-κB activation [11].

To gain more insight into how IKK is activated, we performed yeast two-hybrid screening for proteins interacting with the C-terminal domain of IKKγ. These screenings identified CSN3, a component of the COP9 signalsome [15–18], as a specific IKKγ-interacting protein. Furthermore, our findings suggest that CSN3 is a specific inhibitor of TNF- but not IL-1-induced NF-κB activation.

2. Materials and methods

2.1. Reagents and cell line

Recombinant human TNF and IL-1 (R&D Systems), the anti-Flag (Sigma) and anti-hemagglutinin (HA) (Covance) monoclonal antibodies were purchased from the indicated manufacturers. The human embryonic kidney 293 cell line was a gift from Dr. Zhaodan Cao (Tularik, Inc.), and was maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

2.2. Plasmids

To construct mammalian expression plasmids for IKKγ, its mutants, and CSN3, the inserts were amplified by PCR from a mixture of cDNA libraries (Clontech), and cloned into the pRK-Flag and pRK-HA vectors [19,20]. Antisense CSN3 plasmid was constructed by inserting the CSN3 full-length cDNA into pcDNA3.1(−) (Invitrogen) in reverse orientation.

The NF-κB luciferase reporter construct was a gift from Dr. Gary Johnson (National Jewish Medical and Research Center). The mammalian expression plasmids pRK-myc-TNFR-1, pRK-HA-TRAF2, pRK-HA-RIP, pRK-myc-NIK, p65, pRK-Flag-TRAF6 and crmA were provided by Dr. David Goeddel (Tularik Inc.).

2.3. Yeast two-hybrid screenings

The cDNA encoding the leucine-zipper motif-containing C-terminal domain of IKKγ (amino acids 297–419) was inserted in frame into the Gal4 DNA-binding domain vector pGBT9 (Clontech). The human leukocyte and B cell two-hybrid cDNA libraries were from Clontech. The isolation of positive clones and subsequent two-hybrid interaction analyses were carried out according to the MATCH maker two-hybrid system protocol (Clontech). The identities of the positive clones were analyzed by sequencing.

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2.4. Luciferase reporter gene assays

293 cells ($\sim 2\times10^5$) were seeded on 6 well (35 mm) dishes. Cells were transfected the following day by the standard calcium phosphate precipitation method [21] with 0.5 μg of NF-κB-luciferase reporter construct and various amounts of testing plasmids. Within the same experiment, each transfection was performed in triplicate, and where necessary, enough empty control plasmid was added to keep each transfection receiving the same amount of total DNA. To normalize for transfection efficiency and protein amount, 0.3 μg of RSV-β-galactosidase plasmid was added to all transfections. Luciferase reporter assays were performed using a luciferase assay kit (Pharmingen) and following the manufacturer's protocols. β-Galactosidase activity was measured using the Galacto-Light chemiluminescent kit (Tropix Inc.). Luciferase activities were normalized on the basis of β-galactosidase expression levels. Data shown are averages and standard deviations from one of the representative experiments in which each transfection was performed in triplicate.

2.5. Immunoprecipitation and Western blot

293 cells ($\sim 3\times10^6$ per 100 mm dish) were transfected with the indicated amounts of plasmids by the calcium phosphate method [21]. Thirty-six hours after transfection, cells were collected by centrifugation and lysed in 0.5 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 2 mM sodium pyrophosphate, 2 mM Na_3VO_4) for 30 min. Cell lysates were incubated with 1 μg of anti-Flag or control mouse IgG and 25 μl of a 1:1 slurry of Gamma Bind-G plus Sepharose (Amersham Pharmacia) at 4°C for 3 h. The Sepharose beads were washed three times with 1 ml of lysis buffer. The precipitates were analyzed by Western blot with indicated antibodies.

3. Results

It has been suggested that the N-terminal coiled-coil domain of IKKγ interacts with IKKα and IKKβ, while the C-terminal leucine-zipper-containing domain may interact with upstream components of the NF-κB activation pathways [11,12]. To identify additional proteins that regulate IKK, we performed yeast two-hybrid screenings for proteins interacting with the C-terminal domain of IKKγ (amino acids 297–419). Using IKKγ (amino acids 297–419) as bait, we screened $\sim 1\times10^7$ clones from a human B cell cDNA library and a human leukocyte cDNA library. Among 18 positive clones obtained, two (one from each library) encode CSN3, a protein originally identified as a component of the COP9 signaling complex [15–18].

To determine whether full-length IKKγ interacts with full-length CSN3 in mammalian cells, we constructed mammalian expression plasmids for HA-tagged CSN3 and Flag-tagged

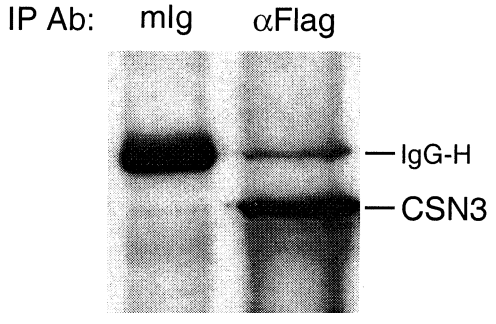

Fig. 1. IKKγ interacts with CSN3 in mammalian cells. 293 cells ($\sim 5\times10^6$) were cotransfected with pRK-Flag-IKKγ (6 μg) and pRK-HA-SGN3 (8 μg). Twenty-four hours after transfection, cells were lysed and immunoprecipitated with a monoclonal anti-Flag antibody or control mouse IgG. The immunoprecipitates were analyzed by Western blot with anti-HA antibody.

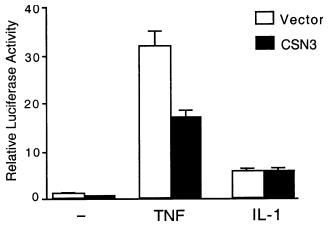

Fig. 2. CSN3 inhibits TNF- but not IL-1-induced NF-κB activation. 293 cells ($\sim 2\times10^5$) were transfected with 0.3 μg of NF-κB-luciferase reporter plasmid, 0.3 μg of RSV-β-galactosidase plasmid and 1 μg of pRK-Flag-CSN3. Sixteen hours after transfection, cells were treated with 20 ng/ml of TNF, 10 ng/ml of IL-1, or left untreated for 8 h. Luciferase activity was measured and normalized on the basis of β-galactosidase levels. Data shown are relative luciferase activity compared with the control transfection.

IKKγ. These plasmids were transfected into 293 cells. Co-immunoprecipitation and Western blot experiments indicate that IKKγ can physically interact with CSN3 in mammalian cells (Fig. 1).

We next determined whether CSN3 can activate NF-κB by reporter gene assays. As shown in Fig. 2, overexpression of CSN3 could not activate NF-κB. However, overexpression of CSN3 significantly inhibited TNF-induced NF-κB activation. In contrast, CSN3 did not affect IL-1-induced NF-κB activation (Fig. 2).

We further examined the effect of CSN3 deficiency on TNF- and IL-1-induced NF-κB activation. To do this, we made a CSN3 antisense construct and transiently transfected it into 293 cells. Western blot experiments indicated that the expressed antisense CSN3 mRNA could partially inhibit expression of CSN3 protein (Fig. 3A). Consistent with an inhibitory role of CSN3 in TNF-induced NF-κB activation, transfection of the antisense CSN3 construct potentiated TNF-induced NF-κB activation (Fig. 3B).

Previously, it has been shown that the signaling molecules TRAF2 and RIP function upstream of IKK and are involved in the TNF-R1- but not IL-1 receptor-mediated NF-κB activation pathway [22–25], whereas TRAF6 is involved in IL-1

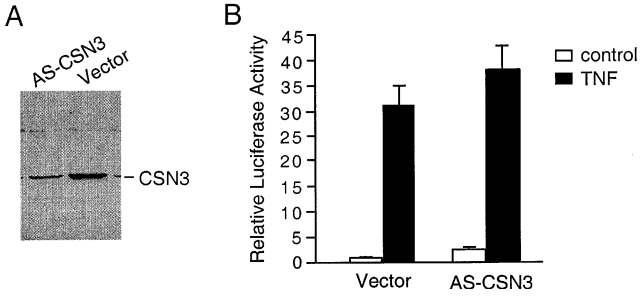

Fig. 3. Down-regulation of CSN3 potentiates TNF-induced NF-κB activation. A: Down-regulation of CSN3 protein by CSN3 antisense construct. 293 cells were transfected with 1 μg of pRK-Flag-CSN3 plasmid together with 8 μg of either empty control plasmid or CSN3 antisense construct. Twenty-four hours after transfection, the cells were lysed and Western blot was performed with anti-Flag antibody. B: Potentiation of TNF-induced NF-κB activation by CSN3 antisense construct. 293 cells ($\sim 2\times10^5$) were transfected with 0.3 μg of NF-κB-luciferase reporter plasmid, 0.3 μg of RSV-β-galactosidase plasmid and 1 μg of indicated plasmids. Sixteen hours after transfection, cells were treated with 20 ng/ml of TNF or left untreated for 8 h. Luciferase activity was then measured and normalized on the basis of β-galactosidase levels. Data shown are relative luciferase activity compared with the control transfection.

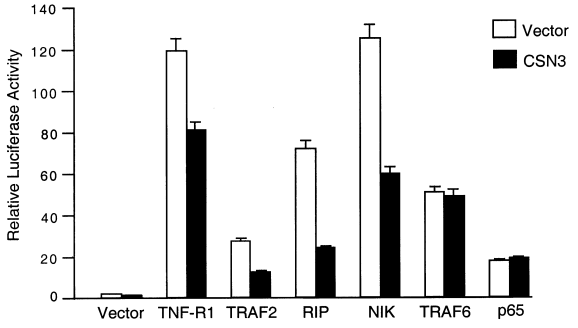

Fig. 4. CSN3 inhibits TRAF2-, RIP-, and NIK-, but not TRAF6-induced NF-κB activation. 293 cells ($\sim 2\times 10^5$) were transfected with 0.3 μg of NF-κB-luciferase reporter plasmid, 0.3 μg of RSV-β-galactosidase plasmid, 0.5 μg of the indicated plasmids, and 2.5 μg of pRK-Flag-CSN3 or empty control plasmid. 1 μg of crmA expression plasmid was added to protect cells from RIP-induced apoptosis. Thirty hours later, luciferase activity was measured and normalized on the basis of β-galactosidase levels. Data shown are relative luciferase activity compared with the control treatment.

receptor but not TNF-R1 signaling [24,25]. We found that overexpression of CSN3 could inhibit TNF-R1-, TRAF2-, RIP- and NIK-, but not TRAF6-induced NF-κB activation (Fig. 4). Taken together, these data suggest that CSN3 can specifically inhibit TNF- but not IL-1-induced NF-κB activation.

4. Discussion

It has been shown that IKKγ is critically involved in activation of NF-κB. Using the yeast two-hybrid system, we have identified CSN3 as a specific IKKγ-interacting protein. Human CSN3 was originally identified as one of the eight subunits in a complex called JAB1 (Jun activation domain-binding protein) containing signalsome [15–18]. This signalsome was subsequently shown to be identical with the human COP9 complex [11,16]. The evolutionarily conserved COP9 complex was originally identified as a repressor of light-controlled development in *Arabidopsis thaliana* [15–18]. The purified human COP9 is a 450 kDa complex that can phosphorylate IκBα and p105 [16]. In addition, CSN3 is also observed in two or three larger complexes with molecular masses of up to approximately 1000 kDa in the lysates of mammalian cells [16]. Since the IKK complex is approximately 900 kDa, which is close to 1000 kDa, our finding that IKKγ interacts with CSN3 suggests the possibility that IKKγ and CSN3 exist in the same signaling complex.

In vitro translation experiments have shown that IKKγ can interact with IKKβ but not with IKKα [11,13]. However, IKK complexes in IKKβ-deficient cells contain IKKα associated with IKKγ [26–28]. N-terminal deletion mutants of IKKγ have suggested that the critical site of interaction with IKK is confined to residues 135–235 [11,12]. Consistent with this is the observation that C-terminal deletion mutants of IKKγ lacking the final 119 [11] or 154 [12] amino acids are able to interact with IKK.

In addition to IKKα and IKKβ, several other IKKγ-interacting proteins have recently been reported. Among them one group are viral proteins, such as adenoviral E3-14.7, [29], equine herpesviral vCLAP [30] and HTLV-1 Tax [31]. Another group are cellular proteins including RIP, A20 and CIK/ActI [32,33]. It has been shown that all three viral proteins bind to the C-terminal region of IKKγ [29–31], while the cellular protein RIP, A20, and CIK/ActI bind to the intermediate region (amino acids 95–218) of IKKγ [32,33]. Our results suggest that CSN3 binds to the C-terminal leucine-zipper-containing domain (amino acids 297–419) of IKKγ. Previously, it has been speculated that the C-terminal domain of IKKγ may be responsible for linking the IKK complex to upstream components of the NF-κB activation pathways [11]. It is possible that inhibition of TNF-induced NF-κB activation may be due to competition of CSN3 with the upstream signaling components for binding to IKKγ.

Our data suggest that CSN3 specifically inhibits TNF- but not IL-1-induced NF-κB activation. This is surprising because it has been suggested that TNF- and IL-1-induced NF-κB activation pathways converge at IKK [4]. Recently, it has been shown by gene knock-out studies that A20, a protein interacting with IKKγ, can also specifically inhibit TNF- but not IL-1-induced NF-κB activation [34]. The mechanisms responsible for the different effects of CSN3 and A20 on TNF- and IL-1-induced NF-κB activation are not clear at this point.

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