TRIM22 can activate the noncanonical NF-κB pathway by affecting IKKα

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Abstract

Tripartite motif 22 (TRIM22) is involved in various cellular processes. It has been reported that TRIM22 can activate nuclear factor-κB (NF-κB) pathway, but the precise mechanism remains unclear. In this study, we explored the exact role of TRIM22 in activating the NF-κB pathway. Different to tumor necrosis factor-α (TNF-α) induction, we found that the overexpression of TRIM22 could induce the processing of p100 to p52 in HEK293T cells. Furthermore, based on the results of co-immunoprecipitation and co-localization experiments, we demonstrated that TRIM22 could interact with IκB kinase (IKKα) but not IKKβ and could increase the level and phosphorylation of IκKα through its really interesting new gene (RING) and spla-ryanodine receptor (SPRY) domains. These results suggest that TRIM22 is able to activate the noncanonical but not the canonical NF-κB pathway by activating IKKα. This finding will aid our understanding of the biological function of TRIM22.

Introduction

The superfamily of tripartite motif (TRIM)-containing proteins is involved in several biological and antiviral processes. TRIM22 (also called Staf50) was first reported as an interferon (IFN)-inducible and restriction factor for the human immunodeficiency virus type 1 (1,2). More recently, several studies have reported broader antiviral activities of TRIM22 in host defense (3,4). Moreover, this protein also plays important roles in apoptosis, differentiation and transcriptional regulation (5–7). TRIM22 contains a TRIM family conserved really interesting new gene (RING), B-Box and Coiled-Coil domains motif at its N-terminal domain and a special spla-ryanodine receptor (SPRY) region at its C-terminal domain. The RING domain of TRIM22 possesses E3 ubiquitin ligase activity and is required for antiviral activity (4,8,9). The SPRY domain is required for TRIM22 formation in nuclear bodies in MCF7 and HepG2 cells (10,11).

Nuclear factor-κB (NF-κB) constitutes a family of transcription factors involved in inflammatory, immune responses, cell growth, apoptosis and differentiation. Increasing evidence suggests that TRIMs modulate various stages of NF-κB activation (12,13). TRIM20 upregulates NF-κB by inducing p65 translocation (14); TRIM30 inhibits NF-κB by inducing the degradation of TAK1-binding protein (TAB)2 and TAB3, which are components of the adaptor-kinase complex (15); and TRIM27 negatively regulates NF-κB by interacting with and repressing IκB kinases (IKKs) (16). It has also been reported that TRIM22 can activate the NF-κB pathway and that deletion of the RING and SPRY domains impairs the activation of NF-κB by TRIM22, but the precise mechanism underlying this activation remains unclear (13). Canonical NF-κB signaling requires the phosphorylation and subsequent degradation of inhibitor of NF-κB α (IκBα), which is regulated by the activation of IKKβ and IKKγ in the IKK complex, which is always induced by tumor necrosis factor-α (TNF-α). In contrast, the noncanonical pathway requires the processing of another NF-κB protein, namely the transformation of p100 to p52, which is regulated by NF-κB-inducing kinase (NIK) and IKKα and occurs without the degradation of IκBα (17). Different from the canonical NF-κB pathway, the noncanonical way regulates specific immunological processes, including the regulation of differentiation and development of lymphoid organs and cells (18).

The diverse functions of TRIM22 are highly correlated with transcriptional pathways that remain not fully elucidated. In this study, we found that TRIM22 could activate the noncanonical NF-κB pathway in an IKKα-dependent manner in HEK293T cells.

Materials and methods

Constructs

The human TRIM22 gene was cloned from pCMV-SPORT6-TRIM22 (ATCC, Manassas, VA); and three mutants of the
gene were amplified based on a previous report (10). The WT and mutant TRIM22 genes were cloned separately into the mammalian expression vector pcDNA3.1 (+) with an HA-tag added to the N-terminal region. The resulting constructs were designated TRIM22-WT (full-length TRIM22), TRIM22-ΔR (RING-domain-deletion mutant), TRIM22-ΔS (SPRY-domain-deletion mutant) and TRIM22-BC (RING- and SPRY-domains-deletion mutant; B-Box-Coiled-Coil domain). The reporter plasmids NF-κB-lucerase (pNF-κB-luc) and RL-TK-luciferase (pRL-TK-luc) were purchased from Agilent (Santa Clara, CA) and Promega (Madison, WI), respectively. The mammalian expression plasmids for IKKα and IKKβ with a Flag-tag added to the N-terminal region were kindly provided by Dr. Hong-Bing Shu (Wuhan University, Wuhan, China).

Reagents and antibodies
 Mouse monoclonal antibodies (Abs) for Flag and HA tags (Sigma-Aldrich, St. Louis, MO), β-actin (Beyotime, Shanghai, China) and β-gal (Cell Signal Tech, Danvers, MA), rabbit polyclonal Abs for p100/p52, p105/p50, c-Rel, p65 (RelA), RelB, IKKα, IKKβ, phosphorylated-p100 (Ser866/870) and phosphorylated-IκBα (Ser32/36) (Cell Signal Tech), rabbit polyclonal Ab for phosphorylated-IKK alpha (S176/S180) (Source Bioscience, Nottingham, UK), Texas Red-conjugated Affinipure goat anti-mouse IgG, fluorescein isothiocyanate (FITC)-conjugated Affinipure goat anti-rabbit IgG (Invitrogen, Carlsbad, CA), recombinant human-TNF-α (Prospec, East Brunswick, NJ), poloxymethylene (Sigma-Aldrich), rabbit polyclonal Ab against HH3, Hoechst, P0013 lysis buffer, radio immunoprecipitation assay (RIPA) and Prolong Antifade (Beyotime), phosphatase and protease inhibitor cocktails (Roche Applied Science, Indianapolis, IN) were purchased from the indicated companies.

Cell transfection and dual-luciferase assay
 HEK293T cells (1 × 10⁵) were seeded in 24-well dishes and transfected with the corresponding plasmids the following day using Lipofectamine™ 2000 (Invitrogen). An empty control vector plasmid was added during each transfection to ensure that the total DNA was equal among the different experimental systems. To normalize the transfection efficiency, pPL-TK-luc was transfected with pNF-κB-luc into the cells. The cells were harvested at 24 h post-transfection, and the activities of NF-κB-luc were analyzed using the Dual-Luciferase® Reporter Assay System according to the manufacturer’s instructions (Promega).

Cell fractionation and western blot analysis
 HEK293T cells (2 × 10⁶) were seeded in six-well dishes and transfected with the corresponding plasmids the following day. At 24 h post-transfection, the cells were lysed in 250 μl of lysis buffer (P0013, containing 1 mg/ml protease inhibitor mixture and phosphatase inhibitor mixture, pH 7.4) and incubated on ice for 20 min with periodic mixing. The cellular debris was cleared by centrifugation at 15 000 × g and 4 °C for 10 min. The samples were separated by 10% SDS-PAGE and analyzed by western blot (WB) as described previously (19).

To isolate the nuclear and cytoplasmic fractions, the cells were washed with phosphate-buffered saline (PBS) and suspended in hypotonic buffer composed of 0.1% nonidet P-40 (NP-40), 1 M DTT, 1 M KCl, 0.5 M EDTA, 1 M N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid’ (HEPES), 1 mg/ml protease inhibitor mixture and 100 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 15 000 × g and 4 °C for 10 min, the resulting supernatant was considered the cytoplasmic extract. The precipitate was washed twice with hypotonic buffer, suspended and sonicated in RIPA buffer (containing 1 mg/ml protease inhibitor mixture and phosphatase inhibitor mixture; pH 7.4) and centrifuged at 15 000 × g at 4 °C for 10 min, and the supernatant was considered the nuclear extract.

Coimmunoprecipitation
 HEK293T cells (2 × 10⁶) were seeded in six-well dishes and transfected with the corresponding plasmids the following day. At 24 h post-transfection, the cells were lysed in 400 μl of lysis buffer. The lysates were incubated with 25 μl of 1/1 slurry of G-Sepharose beads (Santa Cruz Biotechnology, Dallas, TX) and 1 μg of the indicated Ab or control IgG for 4 h. The sepharose beads were washed four times with 300 μl of lysis buffer, and the precipitates were analyzed by WB.

Immunofluorescence assay
 The immunofluorescence assay (IFA) was performed according to our previous report (20) with slight modification. HEK293T cells cultured on glass coverslips were plunged sequentially into 4% polyoxymethylene for 20–30 min, then washed with PBS, permeabilized with PBS containing 0.3% Triton X-100 for 5 min, blocked with PBS containing 1% bovine serum albumin (BSA) and 10% fetal calf serum (FBS) for 1 h at room temperature (RT) and stained with binding buffer containing 1% BSA and primary Ab (2 μg/ml) for 2 h at RT. The cells were rinsed three times with PBS for 5 min and stained with Texas Red-conjugated Affinipure goat anti-mouse IgG (1/200 dilution) or FITC-conjugated AffiniPure goat anti-rabbit IgG (1/200 dilution) (Invitrogen) for 1 h at RT. For nuclear counterstaining, the cells were incubated with Hoechst (Beyotime) for 25 min at RT. The cells were rinsed with PBS, mounted in Prolong Antifade (Beyotime) and then observed by Perkin Elmer (Waltham, MA) UltraView Spinning disk confocal microscopy using a 100 × plan objective.

Results
 TRIM22 induces activation of the noncanonical NF-κB pathway
 We first confirmed whether TRIM22 could activate the NF-κB pathway. TRIM22, PL-TK-luc and NF-κB-luc were cotransfected into HEK293T cells; 24 h later, the cells were lysed and used to perform dual-luciferase assay. The results showed that TRIM22, similar to TNF-α induction, activated NF-κB in a dose-dependent manner (Figure 1A).

It has been reported that the stimulation of the canonical NF-κB pathway by TNF-α is mediated through the induction of the phosphorylation and subsequent degradation of IκBα.
To determine whether TRIM22 induces NF-κB activation through the canonical pathway, we detected the levels of phosphorylated IκBα and total IκBα in the cells transfected with different concentrations of TRIM22 and in the cells treated (positive control) or not treated (negative control) with TNF-α (10 ng/ml) for 2 h. Overexpression of TRIM22 did not induce phosphorylation and degradation of IκBα, whereas TNF-α did (Figure 1B). It is known that the degradation of IκBα induced by TNF-α leads to the release and nuclear translocation of the canonical NF-κB factors p65 (RelA)/p105 or c-Rel/p105. In addition, p50 is removed from p105 and accumulates accompanied by a decrease in the p105 level. However, different from TNF-α induction, the HEK293T cells transfected with TRIM22 did not exhibit similar changes in these proteins (Figure 1C and D). These results imply that TRIM22 does not activate NF-κB through the canonical pathway.

In the noncanonical NF-κB pathway, RelB is released from the IκBα-like protein p100 and translocates with p52 (produced from its precursor p100) into the nucleus (18). To determine whether TRIM22 induces noncanonical NF-κB activation, we detected the levels of p100/p52 and RelB in whole-cell and nuclear extracts of HEK293T cells transfected with TRIM22. As shown in Figure 1C, the level of p100/p52 increased with an increase in the amount of TRIM22. In addition, the levels of RelB and p52 also increased in the nuclear extract (Figure 1D). These results suggest that, consistent with the characteristics of the noncanonical NF-κB pathway, TRIM22 can induce the processing of p100 to p52 and the translocation of the p52/RelB complex into the nucleus, to activate the noncanonical NF-κB pathway.

TRIM22 interacts with IKKα

It is known that p52 produced from p100 is regulated by IKKα but not IKKβ; thus, we performed coimmunoprecipitation (Co-IP) and IFA assays to determine whether TRIM22 interacts with IKKα or IKKβ. The Co-IP results showed that IKKα, but not IKKβ, could be pulled down by TRIM22 (Figure 2A and B), and the IFA assay results showed that IKKα and TRIM22 are co-localized in the cytoplasm of HEK293T cells (Figure 2B). The above-mentioned results demonstrate that TRIM22 can interact with IKKα.
induces NF-κB (Figure 3B). These results suggest that TRIM22 regulates NF-κB physiological function. To determine which domain of p100 to p52.

Both the RING and SPRY domains of TRIM22 are necessary for inducing NF-κB activation mediated by IKKα

To determine the role of IKKα in the TRIM22-induced noncanonical NF-κB pathway, we detected the levels of IKKα, IKKβ and phosphorylated IKKα (P-IKKα) and p100 (P-p100) in the TRIM22-transfected HEK293T cells. An increase in the expression level of TRIM22 resulted in dose-dependent increases in the levels of IKKα, P-IKKα and P-p100 in the cytoplasmic extract, but the level of IKKβ did not change (Figure 3B). These results suggest that TRIM22 induces NF-κB activation by enhancing the phosphorylation of IKKα and subsequently activating the transformation of p100 to p52.

The domains of TRIM22 play different roles in its physiological function. To determine which domain of TRIM22 regulates NF-κB activity, we performed dual-luciferase and WB assays using a series of deletion mutants of TRIM22 (Figure 3A and C). Compared with the wild type, all mutants of TRIM22 (TRIM22-ΔR, TRIM22-ΔS and TRIM22-BC) were not able to induce NF-κB activation or to increase the levels of P-IKKα and IKKα (Figure 3C). These results suggest that both the RING and SPRY domains are necessary for the induction of NF-κB activation through IKKα.

Discussion

TRIM22 plays diverse roles in the immune response and cell differentiation (21–23). In addition, it has been reported that the transcription factor NF-κB can be activated by TRIM22 (13), but the mechanism underlying this activation remains not well understood. In this article, we found that TRIM22 could activate the noncanonical NF-κB pathway through an IKKα-dependent mechanism.

Our results show that TRIM22 does not affect the components of the canonical pathway but can activate the key component of the noncanonical pathway, namely IKKα. TRIM22 can interact with IKKα, leading to the phosphorylation of IKKα and p100, and then induces the transformation of p100 to p52 and the formation of the RelB/p52 complex. This complex translocates into the nucleus to activate NF-κB (Figure 4).

It has been shown that NIK is important for the phosphorylation of IKKα (24). However, the endogenous level of NIK in most cell lines is too low to be detected by WB (18). To determine whether NIK is one of the upstream kinases of IKKα, we detected the levels of NIK and TNF receptor associated factor 3 (TRAF3) in the TRIM22-overexpressing cells because TRAF3 is crucial for the turnover of NIK (25). We observed that an increase in the level of TRIM22 resulted in a decrease in the expression level of TRAF3 (supplementary information), but the level of NIK could not be detected (data not shown). Interestingly, once p100 processing is activated by the human T-cell leukemia virus oncoprotein Tax in T cells, the activation of IKKα relies on IKKγ and not NIK (26). Therefore, two possibilities could explain our results: (1) IKKα is phosphorylated by NIK, but the level of NIK may be too low to be detected; or (2) IKKα is phosphorylated by other kinases that interact with IKKα and TRIM22, and TRIM22 activates these unknown kinases. Further experiments are required to distinguish among these possibilities.

With the exception of its anti-viral ability, TRIM22 may play roles in the complex network of cellular regulation. A few studies have demonstrated that TRIM22 plays important roles in cell differentiation and proliferation (1,23). TRIM22 is a novel p53 target gene that is directly activated by p53 (22). In IFN-gamma-induced TRIM22 expression, p300, the homology protein of p300/CREB-binding protein (CBP), is the main transcription co-activator and collaborates with IRF-1 to stimulate TRIM22 expression (5). In this study, we showed that the overexpression of TRIM22 could activate the noncanonical NF-κB pathway. The mechanism through which TRIM22 mediates the p53, IFN and NF-κB crosstalk remains to be determined. In our study, the role of IKKα in the TRIM22-induced noncanonical NF-κB pathway may provide some insights into this question. A previous study, which supports our results, showed that IKKα induces CBP phosphorylation to regulate crosstalk between p53 and
Moreover, p300, the homology protein of CBP, also govern NF-κB transcription in an IKKα-mediated manner (28). In contrast, although direct evidence supporting the IFN-γ-induced activation of the noncanonical NF-κB pathway has not yet been identified, a previous study showed that IFNα/βstimulates an alternative NF-κB pathway and induces the processing of p100 into p52 (29). The correlation between IKKα and TRIM22 has been reviewed extensively, and the function of TRIM22 may be closely linked to the regulation of IKKα.

Collectively, our results reveal a novel role for IKKα in the activation of the noncanonical NF-κB pathway by TRIM22, and the mechanism underlying this activation may be helpful for extending our understanding of the biological function of TRIM22.

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Declaration of interest

The authors report no declarations of interest.

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