TRIP6 is a RIP2-associated common signaling component of multiple NF-κB activation pathways

Lianyun Li[1], Liang-Hua Bin[2], Fu Li[1], Yi Liu[1], Danying Chen[1], Zhonghe Zhai[1] and Hong-Bing Shu[1,2,*]

[1]Department of Cell Biology and Genetics, College of Life Sciences, Peking University, Beijing 100871, China
[2]Department of Immunology, National Jewish and Research Center, University of Colorado Health Sciences Center, 1400 Jackson Street, Denver, CO 80206, USA
*Author for correspondence (e-mail: shuh@njc.org)

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Summary

Receptor-interacting protein 2 (RIP2) is a member of the RIP kinase family that has been shown to be crucially involved in inflammation, innate and adaptive immune responses. The physiological and pathological roles of RIP2 are mediated through its involvement in multiple NF-κB activation pathways, including those triggered by tumor necrosis factor (TNF), interleukin 1 (IL-1), Toll-like receptor 2 (TLR2), TLR3, TLR4 and Nod1. In this report, we identified the LIM-domain-containing protein TRIP6 as a RIP2-interacting protein in yeast two-hybrid screens. In mammalian cells, TRIP6 interacts with RIP2 in a TNF- or IL-1-dependent manner. Overexpression of TRIP6 potentiates RIP2-mediated NF-κB activation in a dose-dependent manner. The LIM domains of TRIP6 are responsible for its interaction with RIP2. TRIP6 also interacts with TRAF2, a protein that is crucially involved in TNF signaling, as well as the IL-1 receptor, TLR2 and Nod1. Overexpression of TRIP6 potentiates NF-κB activation by TNF, IL-1, TLR2 or Nod1, whereas a dominant negative mutant or RNA-interference construct of TRIP6 inhibits NF-κB activation by TNF, IL-1, TLR2 or Nod1. Moreover, TRIP6 also potentiates RIP2- and Nod1-mediated ERK activation. These data have established a physical and functional association between TRIP6 and RIP2, and suggest that RIP2's involvement in multiple NF-κB and ERK activation pathways is mediated through TRIP6.

Key words: RIP2, TRIP6, NF-κB, TNF, IL1, Nod1

Introduction

Receptor-interacting protein 2 (RIP2), also called RICK/CARDIAK, is a serine/threonine protein kinase belonging to the RIP kinase family (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998). This family of kinases includes RIP, RIP2, RIP3 and RIP4 (Stanger et al., 1995; Meylan et al., 2002; Yu et al., 1999; Sun et al., 1999). These kinases share significant similarity in their N-terminal kinase domains but possess structurally divergent C-terminal domains. RIP2 contains a caspase activation and recruitment domain (CARD) at its C-terminus (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998). It has been shown that RIP2 interacts with multiple proteins including TRAF1, TRAF5, TRAF6, Casper/c-FLIP/CLARP, caspases 1 and 8, and cIAP1, and is recruited to multiple receptor signaling complexes including tumor necrosis factor receptor 1 (TNF-R1), CD40 and Toll-like receptor 2 (TLR2) (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998; Kobayashi et al., 2002). Overexpression of RIP2 leads to activation of the transcription factor NF-κB and the kinases JNK and ERK (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998). Overexpression of RIP2 also induces apoptosis or potentiates Fas- and caspase-8-induced apoptosis (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998). These studies suggest that RIP2 plays multiple roles in several signaling pathways.

The definitive experiments on the physiological and pathological roles of RIP2 come from two independent mouse gene-knockout studies (Kobayashi et al., 2002; Chin et al., 2002). It was shown that RIP2-deficient macrophages had decreased activation of NF-κB, JNK, p38 and ERK upon treatment with lipopolysaccharide (LPS) (a TLR4 ligand), whereas RIP-2 deficient mice were resistant to the lethal effects of LPS-induced endotoxic shock (Kobayashi et al., 2002; Chin et al., 2002). Production of the inflammatory cytokines interleukin 6 (IL-6) and tissue necrosis factor (TNF), and the chemokine IP10 was severely reduced in RIP2-deficient macrophages or fibroblasts on stimulation with LPS or lipoteichoic acid (ligands for TLR4), polyinosinic-polycytidylic acid (a ligand for TLR3) or peptidoglycan (a ligand for TLR2) but not with CpG DNA (a ligand for TLR9) (Kobayashi et al., 2002; Chin et al., 2002). RIP2 deficiency also impaired IL-1-mediated T cell co-stimulation and IL-18-induced interferon γ (IFN-γ) production by natural killer cells (Kobayashi et al., 2002). These studies suggest that RIP2 is crucially involved in inflammatory response and innate immunity mediated by TLR2, TLR3, TLR4, IL-1 and IL-18 but not TLR9.

In addition to TLRs, Nod-family proteins have also been implicated in innate immunity (Inohara and Nunez, 2003). The cytoplasmic Nod proteins contain three domains: a N-terminal CARD domain, a nucleotide-binding domain (NBD) and C-terminal leucine-rich repeats (LRRs). These proteins are

homologous to the NBD-LRR-like disease-resistance proteins in plants and are involved in the detection of cytoplasmic pathogens through their LRRs (Inohara and Nunez, 2003). The prototypic member of the Nod protein family, Nod1, is activated on infection of *Shigella flexneri* and in turn potently activates NF-κB (Inohara and Nunez, 2003; Inhohara et al., 1999; Inohara et al., 2000). It has been shown that Nod1 interacts with RIP2 and that Nod1-mediated NF-κB activation is completely absent from RIP2-deficient cells, suggesting that RIP2 is also required for Nod1-mediated innate immunity against cytoplasmic pathogens (Kobayashi et al., 2002; Chin et al., 2002; Inohara and Nunez, 2003; Inhohara et al., 1999; Inohara et al., 2000).

RIP2-deficient T cells showed severely reduced NF-κB activation, IL-2 production and proliferation on T-cell receptor (TCR) engagement, and impaired differentiation to class-1 T-helper (T_{h1}) cells, suggesting that RIP2 is required for optimal TCR signaling and T-cell differentiation and is involved in adaptive immune systems (Kobayashi et al., 2002).

The gene knockout studies have established RIP2 as a crucial signaling component in multiple pathways involved in inflammation and innate and adaptive immunity. In this study, we identified TRIP6 (Lee et al., 1995), a LIM-domain-containing protein, as a novel RIP2-interacting protein. Our findings suggest that TRIP6 is involved in NF-κB and ERK activation mediated by multiple pathways including TNF, IL-1, TLR2 and Nod1, pointing to a possible mechanism responsible for RIP2's involvement in multiple pathways.

Materials and Methods

Reagents

Recombinant human TNF, IL-1 and IFN-γ (R&D Systems), mouse monoclonal antibody against the Flag and hemagglutinin (HA) epitopes (Sigma), rabbit polyclonal antibody against RIP2 (Santa Cruz Biotechnology), and human embryonic kidney 293 cells (ATCC) were purchased from the indicated sources. Rabbit polyclonal antibody against TRIP6 was kindly provided by M. Beckerle (University of Utah, Salt Lake City, UT) and was purchased from Bethyl Laboratories.

Yeast two-hybrid screening

To construct a RIP2 bait vector, a *RIP2* cDNA fragment was inserted in frame into the Gal4 DNA-binding domain vector pGBT (Clontech). A human B-cell cDNA library (ATCC) was screened as described (Hsu et al., 1996; Huang et al., 2004).

Plasmids

An expression plasmid for human TRIP6 (pcDNA-Flag-hTRIP6) was provided by T. Gilmore (Boston University, MA). Mammalian expression plasmids for HA-tagged TRIP6 and its deletion mutants, Flag-tagged RIP2 and its deletion mutants were constructed by polymerase chain reaction (PCR) amplification of the corresponding cDNA fragments and subsequently cloning into a cytomegalovirus (CMV) promoter-based vector containing a 5′ HA or Flag tag.

Expression plasmids for the interleukin 1 receptor (IL-1R), IL-1R accessory protein (IL-1RAcP), IRAK1, TRAF6 (Z. Cao, Tularik), TRADD, IKKβ (D. Goeddel, Tularik), TLR2 (B. Beutler, Scripps Research Institute), Tollip (L.-G. Xu, National Jewish Medical and Research Center), RICK/RIP2 and Nod1 (G. Nunez, University of Michigan) were provided by the indicated investigators. NF-κB (G. Johnson, University of North Carolina) and interferon response factor 1 (IRF-1) (U. Schindler, Tularik) luciferase reporter plasmids were provided by the indicated investigators.

A human TRIP6 RNA interference (RNAi) plasmid was constructed by inserting the target sequence GCCTGGACGCCGA-GATAGA into the pSuper.Retro vector (OligoEngine) at *Bgl*II/*Hin*dIII sites.

Reporter gene assays

293 cells (~1×10^5) were seeded on 12-well dishes and transfected the following day by the standard calcium phosphate precipitation. Within the same experiment, each transfection was performed in duplicate and, where necessary, empty control plasmid was added to ensure that each transfection to receive the same amount of total DNA. To normalize for transfection efficiency, 0.1 μg pTK-RL plasmid (encoding *Rellina* luciferase) was added to each transfection. Dual specific luciferase reporter gene assays were performed using a kit (Promega). Firefly luciferase activities were normalized on the basis of *Renilla* luciferase activities. All reporter-gene assays were repeated at least three times; data shown are averages from one representative experiment. The standard errors were less than 10% of the average values for all samples.

Coimmunoprecipitation and western blot analysis

293 cells (~2×10^6) were transfected by calcium-phosphate precipitation. Transfected cells were lysed in 1 ml lysis buffer [20 mM Tris.HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride]. For each immunoprecipitation, 0.4 ml aliquots of lysates were incubated with 0.5 μg of the indicated antibody or control IgG, and 25 μl of 1:1 slurry of GammaBind-G Sepharose (Amersham Pharmacia) for at least 1 hour. The Sepharose beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were fractionated by SDS-PAGE and subsequent western blot analyses were performed.

Results

Identification of TRIP6 as a RIP2-associated protein by yeast two-hybrid screens

To identify RIP2-associated proteins unambiguously, we performed yeast two-hybrid screenings with RIP2 as bait. We screened a total of ~2×10^6 clones from a human B-cell library and obtained 11 β-galactosidase-positive clones. One of the clones encodes for a C-terminal fragment (amino acids 253-480) of TRIP6, which was originally identified as a thyroid hormone receptor interacting cytoplasmic protein (Lee et al., 1995).

RIP2 interacts with TRIP6 in mammalian overexpression system

To determine whether RIP2 interacts with TRIP6 in mammalian cells, we transfected 293 cells with expression plasmids for HA-tagged TRIP6 and Flag-tagged RIP2, and performed co-immunoprecipitation experiments. The results indicated that RIP2, but not RIP, interacted with TRIP6 (Fig. 1B).

Domain mapping of interaction between RIP2 and TRIP6

RIP2 contains an N-terminal kinase domain, an intermediate

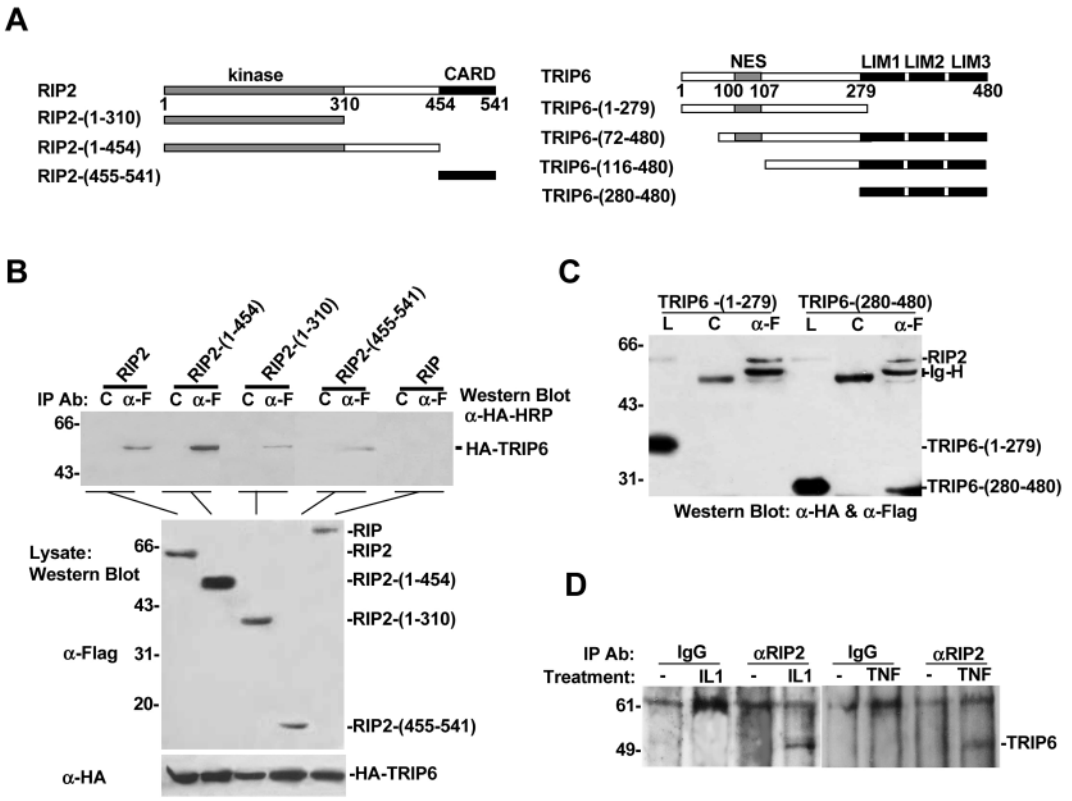

Fig. 1. RIP2 interacts with TRIP6. (A) The structures of RIP2, TRIP6 and their deletion mutants. The kinase and CARD domains of RIP2 and the LIM domains and nuclear export sequence (NES) of TRIP6 are indicated. (B) TRIP6 interacts with RIP2 and its individual domains. 293 cells (2×10^6) were transfected with 5 μg each of HA-TRIP6 and Flag-tagged RIP2 or its mutant plasmids. Cell lysates were immunoprecipitated with anti-Flag antibody (α-F) or control mouse IgG (C). The immunoprecipitates were analysed by western blot with horseradish-peroxidase (HRP)-conjugated anti-HA antibody (top). Expression of the transfected proteins in the lysates was detected by western blot analysis with anti-Flag (middle) or anti-HA (bottom) antibody. (C) RIP2 interacts with the LIM domains of TRIP6. 293 cells (2×10^6) were transfected with 5 μg each of Flag-RIP2 and HA-tagged TRIP6 and its mutant plasmids. Cell lysates were immunoprecipitated with anti-Flag antibody (α-F) or control mouse IgG (C). The immunoprecipitates and cell lysates (L) were analysed by western blot with a combination of anti-HA and anti-Flag antibodies. (D) RIP2 and TRIP6 interact in untransfected cells in a stimulation-dependent manner. 293 cells (2×10^7) were treated with TNF (10 ng ml^{-1}) or IL-1 (10 ng ml^{-1}), or left untreated for 5 minutes. Cells were lysed and the lysate was immunoprecipitated with goat anti-RIP2 antibody or control goat IgG. The immunoprecipitates were analysed by western blot with rabbit anti-TRIP6 antibody.

domain and a C-terminal CARD domain (Fig. 1A). TRIP6 contains three LIM domains at its C-terminus and a nuclear export sequence (NES) at its N-terminus (Fig. 1A). To determine which domain of RIP2 interacts with TRIP6, we made three deletion mutants of RIP2 including amino acids 1-310 (kinase domain), 1-454 (kinase and intermediate domains) and 455-541 (CARD domain). Transient transfection and co-immunoprecipitation experiments indicated that both the kinase and the CARD domains of RIP2 could interact with TRIP6 (Fig. 1B). Previously, it has been shown that both the kinase and the CARD domains are required for RIP2-induced NF-κB activation and apoptosis, suggesting that integrity of the molecule is essential for its signaling (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998).

The TRIP6 clone we identified from yeast two-hybrid screening contains the C-terminal LIM domains, suggesting that the LIM domains of TRIP6 are sufficient for interacting with RIP2. This was confirmed by co-immunoprecipitation experiments in mammalian cells. TRIP6-(280-480), a TRIP6 deletion mutant containing the three LIM domains, interacted with RIP2 (Fig. 1C). In these experiments, the N-terminal domain of TRIP6 (amino acids 1-279) did not interact with RIP2 (Fig. 1C).

RIP2 interacts with TRIP6 in a stimulation-dependent process in untransfected cells

To determine whether endogenous RIP2 is associated with TRIP6 in untransfected cells, we performed endogenous co-immunoprecipitation experiments. As shown in Fig. 1D, RIP2 and TRIP6 do not interact in unstimulated 293 cells, but are associated with each other upon IL-1 or TNF stimulation. These results suggest that RIP2 and TRIP6 interact in a stimulation-dependent manner.

TRIP6 synergizes with RIP2 to activate NF-κB

Because RIP2 is involved in NF-κB activation by multiple pathways (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998; Kobayashi et al., 2002; Chin et al., 2002) and RIP2

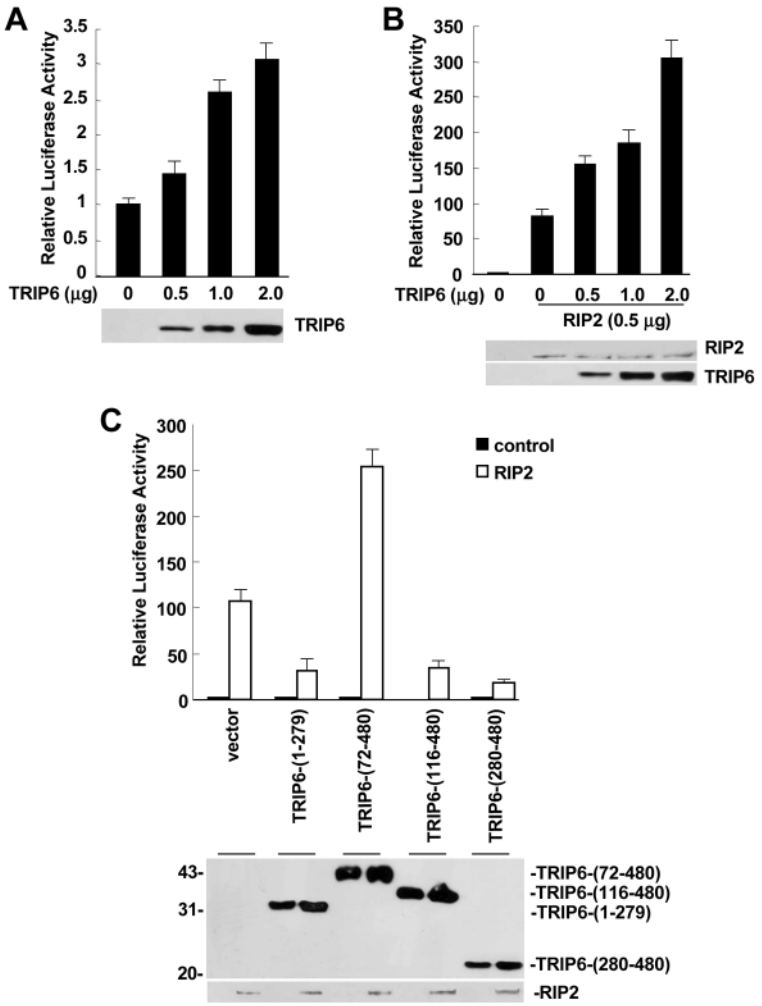

Fig. 2. TRIP6 is involved in RIP2-mediated NF-κB activation. (A) TRIP6 weakly activates NF-κB in a dose-dependent manner. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid and the indicated amounts of HA-tagged TRIP6 expression plasmid. Luciferase assays were performed 14 hours after transfection. TRIP6 expression levels in the transfected cells were analysed by western blot with anti-HA antibody (bottom). (B) TRIP6 synergizes with RIP to activate NF-κB. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid, 0.5 μg Flag-RIP2 expression plasmid and the indicated amounts of HA-TRIP6 expression plasmid. Luciferase assays were performed 14 hours after transfection. Increased TRIP6 expression did not affect RIP2 levels as suggested by western blots with anti-Flag and anti-HA antibodies (bottom). (C) Effects of TRIP6 deletion mutants on RIP2-mediated NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid, 0.5 μg Flag-RIP2 expression plasmid (empty bars) or empty control plasmid (filled bars), and 0.5 μg of plasmids encoding the indicated HA-tagged TRIP6 mutant. Luciferase assays were performed 14 hours after transfection. Expression levels of RIP2 and the TRIP6 mutants were analysed by western blots with anti-Flag and anti-HA antibodies respectively (bottom).

interacts with TRIP6, we determined whether TRIP6 is also involved in NF-κB activation by reporter-gene assays. The results indicated that overexpression of TRIP6 could weakly activate NF-κB in a dose-dependent manner (Fig. 2A). In addition, TRIP6 could synergize with RIP2 to activate NF-κB (Fig. 2B).

We also determined the effects of various TRIP6 mutants on RIP2-induced NF-κB activation. As shown in Fig. 2C, deletion of the N-terminal 71 amino acid had no effect on the ability of TRIP6 to synergize with RIP2 to activate NF-κB. However, further deletion of the NES motif or deletion of the C-terminal LIM domains converted TRIP6 into dominant-negative mutants that potently inhibited RIP2-induced NF-κB activation (Fig. 2C). These results indicated that TRIP6 is functionally associated with RIP2.

TRIP6 is involved in TNF-induced NF-κB activation

TRIP6 interacts with RIP2 in a TNF-dependent process, suggesting that TRIP6 is involved in TNF signaling. We determined whether TRIP6 is associated with components of the TNF-R1 signaling pathway. In transient transfection and co-immunoprecipitation experiments, TRIP6 interacted with TRAF2 but not TRADD and IKKβ (Fig. 3). TRIP6 also did not interact with TNF-R1 (data not shown). These results indicated that TRIP6 participates in TNF signaling through association with TRAF2. Consistent with these observations, TRIP6 potentiated TNF-induced NF-κB activation in a dose-dependent manner (Fig. 4A) but had no significant effects on IFN-γ-induced IRF-1 activation (Fig. 4B). In addition, a TRIP6 deletion mutant [either TRIP6-(280-480) or TRIP6-(1-279)] dramatically inhibited NF-κB activation induced by TNF (Fig. 4C) as well as overexpression of TRAF2 and TRADD (Fig. 4D).

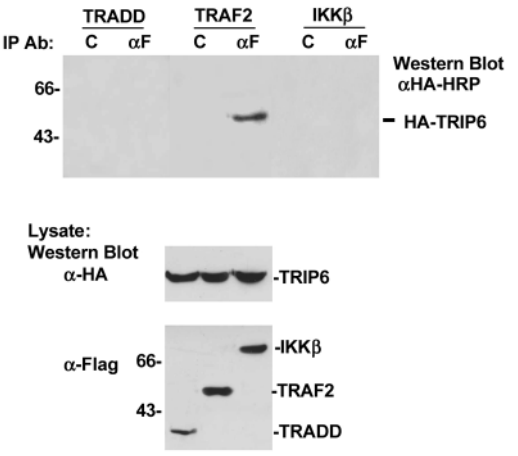

Fig. 3. TRIP6 interacts with TRAF2. 293 cells (2×10^6) were transfected with 5 μg each of plasmids encoding HA-TRIP6 or Flag-tagged TRADD, TRAF2 or IKKβ. Cell lysates were immunoprecipitated with anti-Flag antibody (α-F) or control mouse IgG (C). The immunoprecipitates were analysed by western blot with horseradish-peroxidase (HRP)-conjugated anti-HA antibody. Expression of the transfected proteins in the lysates was comparable, as indicated by western blot analysis with anti-Flag and anti-HA antibodies (bottom).

TRIP6 is involved in IL-1-, TLR2- and Nod1-induced NF-κB activation

Previous studies have demonstrated an essential role for RIP2 in IL-1, TLR2, TLR3, TLR4 and Nod1 signaling (Kobayashi et al., 2002; Chin et al., 2002). We determined whether TRIP6 is similarly involved in signaling in these pathways. We first

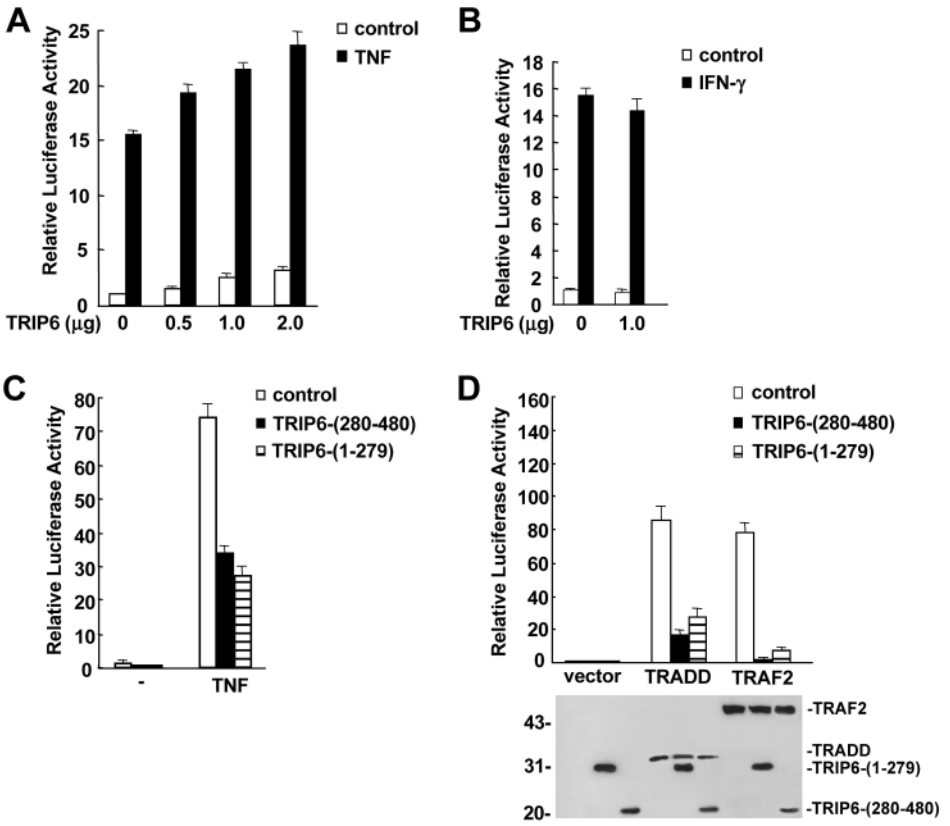

Fig. 4. TRIP6 is involved in TNF-induced NF-κB activation. (A) TRIP6 potentiates TNF-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid and the indicated amounts of TRIP6 expression plasmid. 14 hours after transfection, cells were treated with TNF (10 ng ml^{-1}) (filled bars) or left untreated (empty bars) for 6 hours before luciferase assays were performed. (B) TRIP6 has no significant effect on IFN-γ-induced IRF-1 activation. 293 cells (1×10^5) were transfected with 0.1 μg IRF-1 luciferase reporter plasmid and 1 μg TRIP6-encoding or control plasmid. 14 hours after transfection, cells were treated with IFN-γ (100 ng ml^{-1}) (filled bars) or left untreated (empty bars) for 6 hours before luciferase assays were performed. (C) TRIP6 mutants inhibit TNF-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid and 0.5 μg TRIP6-(280-480)-encoding (filled bars), TRIP-(1-279)-encoding (dashed bars) or empty control (empty bars) plasmid. 14 hours after transfection, cells were treated with TNF (10 ng ml^{-1}) or left untreated, as indicated, for 6 hours before luciferase assays were performed. (D) TRIP6 mutants inhibit TRAF2- and TRADD-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid, 0.5 μg HA-tagged TRIP6-(280-480)-encoding (filled bars), HA-tagged TRIP6-(1-279)-encoding (dashed bars) or empty control (empty bars) plasmid, and 0.5 μg of Flag-tagged TRAF2 or TRADD expression plasmid, as indicated. Luciferase assays were performed 14 hours after transfection. Expression levels of the transfected proteins were analysed by western blot with anti-Flag and anti-HA antibodies (bottom).

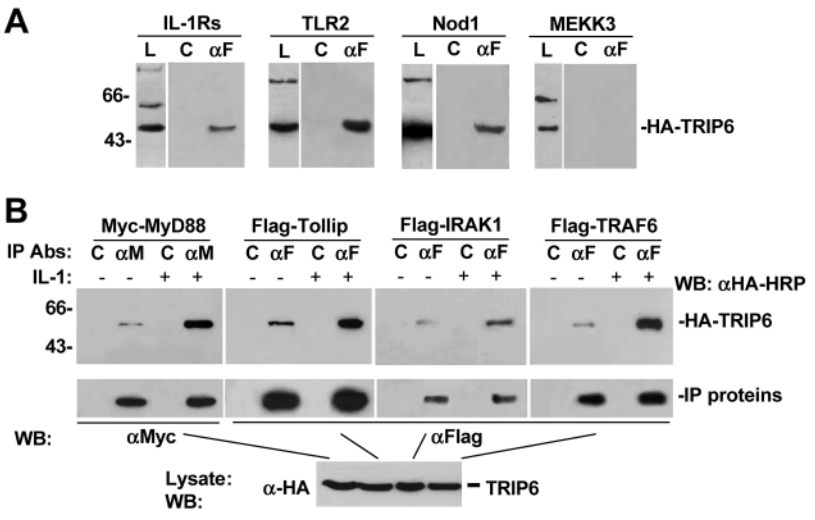

Fig. 5. TRIP6 interacts with signaling components in the IL-1, TLR2 and Nod1 pathways. (A) TRIP6 interacts with IL-1 receptors, TLR2 and Nod1 but not MEKK3. 293 cells (2×10^6) were transfected with 5 μg each of HA-TRIP6 and Flag-tagged IL-1Rs (IL-1R+IL-1RAcP), TLR2, Nod1 or MEKK3. Cell lysates were immunoprecipitated with anti-Flag antibody (αF) or control mouse IgG (C). The immunoprecipitates were analysed by western blots with horseradish-peroxidase (HRP)-conjugated anti-HA antibody (right). Expression of the transfected proteins in the lysates was detected by western blot analysis withanti-Flag and anti-HA antibodies (left). (B) TRIP6 interacts with MyD88, Tollip, IRAK and TRAF6. 293 cells (2×10^6) were transfected with 5 μg each of plasmids encoding HA-TRIP6 and Flag-tagged IRAK, Tollip or TRAF6, or Myc-tagged MyD88, as indicated. Before immunoprecipitation, cells were treated with IL-1 (+) or left untreated (–), as indicated, for 2 minutes. Cell lysates were immunoprecipitated with anti-Flag antibody (αF), anti-Myc (αM) or control mouse IgG (C). The immunoprecipitates were analysed by western blots with HRP-conjugated anti-HA antibody (top). The same blots were detected again with anti-Myc or anti-Flag antibody as indicated (middle). Expression of transfected HA-TRIP6 in the lysates was detected by western blot analysis with anti-HA antibody (bottom).

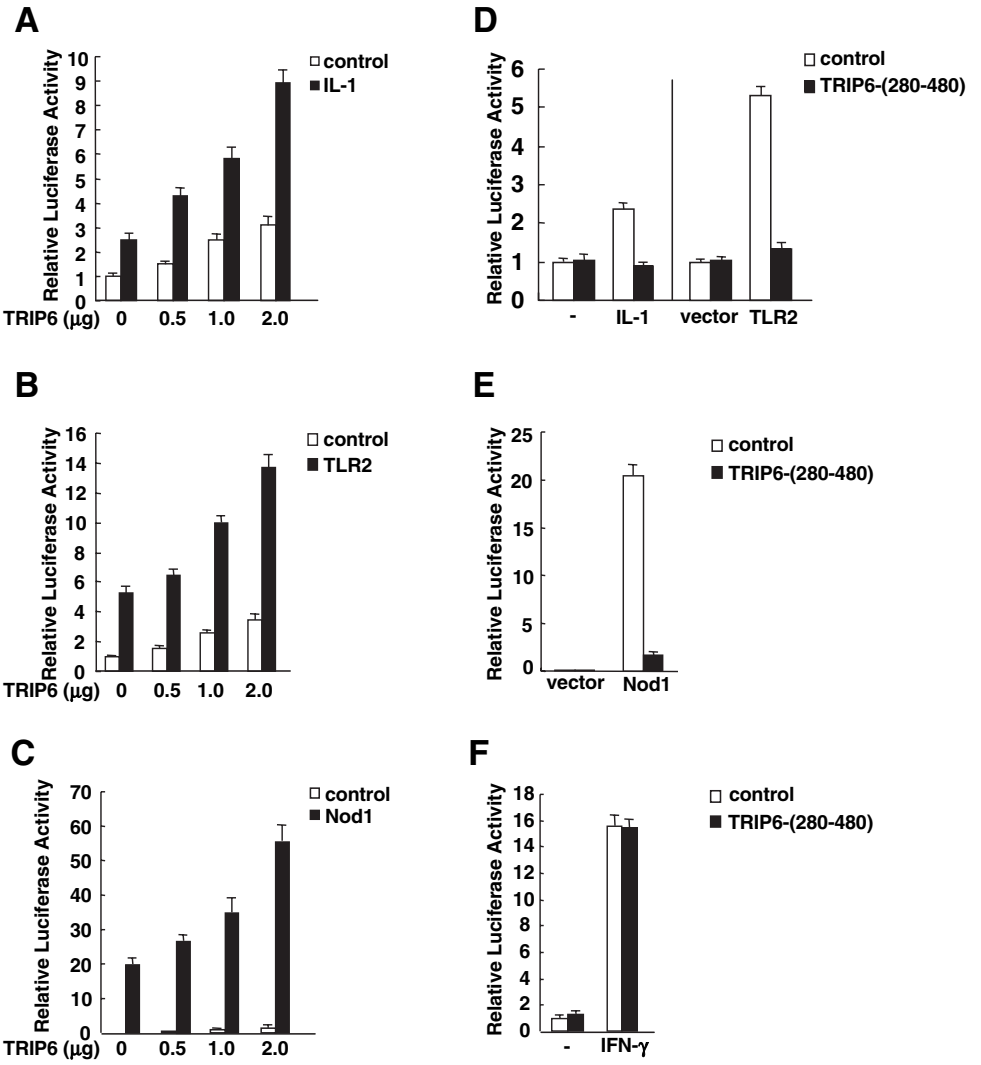

Fig. 6. TRIP6 is involved in IL-1-, TLR2- and Nod1-induced NF-κB activation. (A) TRIP6 potentiates IL-1-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid and the indicated amounts of TRIP6 expression plasmid. 14 hours after transfection, cells were treated with IL-1 (10 ng ml^{-1}) (filled bars) or left untreated (empty bars) for 6 hours before luciferase assays were performed. (B) TRIP6 potentiates TLR2-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid, 0.5 μg TLR2-encoding (filled bars) or empty control (empty bars) plasmid, and the indicated amounts of TRIP6 expression plasmid. Luciferase assays were performed 14 hours after transfection. (C) TRIP6 potentiates Nod1-induced NF-κB activation. The experiments were done as in B except that TLR2 was replaced with 0.1 μg Nod1 expression plasmid. (D,E) A TRIP6 mutant inhibits IL-1-, TLR2- and Nod1-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid and 0.5 μg TRIP6-(280-480)-encoding (filled bars) or empty control (empty bars) plasmid. 14 hours after transfection, cells were treated with IL-1 (10 ng ml^{-1}) or left untreated for 6 hours before luciferase assays were performed (D). Alternatively, 293 cells (1×10^5) were transfected with 0.1 μg NF-κB luciferase reporter plasmid, 0.5 μg TRIP6-(280-480)-encoding (filled bars) or empty control (empty bars) plasmid, and 0.5 μg of TLR2 (D) or Nod1 (E) expression plasmid. Luciferase assays were performed 14 hours after transfection. (F) TRIP6 dominant-negative mutant has no effect on IFN-γ-induced IRF-1 activation. 293 cells (1×10^5) were transfected with 0.1 μg IRF-1 luciferase reporter plasmid and 1 μg TRIP6-(280-476)-encoding (filled bars) or control (empty bars) plasmid. 14 hours after transfection, cells were treated with IFN-γ (100 ng ml^{-1}) or left untreated for 6 hours before luciferase assays were performed.

determined whether TRIP6 interacts with components of these pathways. In transient-transfection and co-immunoprecipitation experiments, TRIP6 interacted with IL-1 receptor complex (IL-1R and IL-1RAcP), TLR2 and Nod1 but not MEKK3 (mitogen-activated protein kinase/ERK kinase kinase 3) (Fig. 5A). TRIP6 also interacted with MyD88, Tollip, IRAK and TRAF6, downstream signaling components of the IL-1 receptors and TLRs (Fig. 5B). Interestingly, the interactions of TRIP6 with these signaling components are increased by IL-1 stimulation (Fig. 5B). In reporter-gene assays, TRIP6 could potentiate IL-1-, TLR2- and Nod1-induced NF-κB activation in a dose-dependent manner (Fig. 6A-C). The TRIP6 mutant TRIP6-(280-480) potently inhibited IL-1-, TLR2- and Nod1-induced NF-κB activation (Fig. 6D,E). Similarly, TRIP6-(1-279) also inhibited IL-1-, TLR2- and Nod1-induced NF-κB activation (data not shown). In these experiments, TRIP6-(280-480) did not inhibit IFN-γ-induced IRF-1 activation (Fig. 6F). These data suggest that TRIP6 is a component of IL-1-, TLR2 and Nod-induced NF-κB activation pathways.

Inhibition of TRIP6 expression downregulates NF-κB activation by TNF, IL1, TLR2 and Nod1

To further determine whether TRIP is involved in NF-κB activation under physiological conditions, we constructed a human TRIP6 RNAi plasmid. This plasmid could significantly inhibit overexpressed TRIP6 in 293 cells (Fig. 7A). Using this plasmid, we established a 293 cell line that stably expresses human TRIP6 RNAi. As shown by western blot analysis, endogenous TRIP6 was significantly downregulated in TRIP6 RNAi 293 cells in comparison to control RNAi vector-transfected cells (Fig. 7B). In reporter-gene assays with this cell line, we found that TRIP6 RNAi could significantly inhibit NF-κB activation triggered by TNF or IL-1, or by overexpression of TLR2 or Nod1 (Fig. 7C). These data suggest

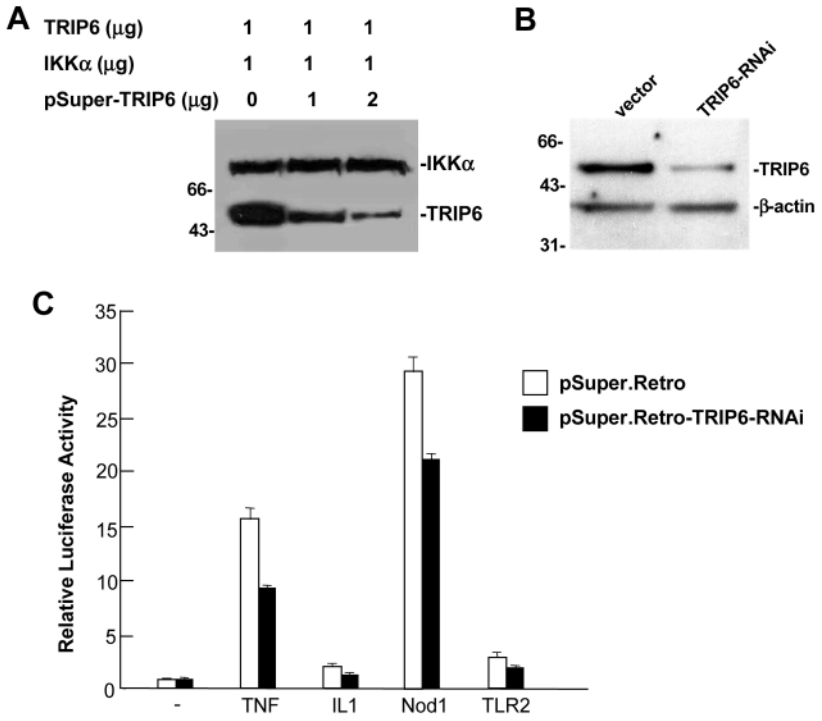

Fig. 7. Effects of TRIP6 RNAi on NF-κB activation by various stimuli. (A) TRIP6 RNAi inhibits the expression of transfected TRIP6. 293 cells (2×10^5) were transfected with 1 μg of an expression plasmid for Flag-TRIP6, 1 μg of an expression plasmid for Flag-IKKα and the indicated amounts of TRIP6 RNAi plasmid (pSuper-TRIP6). 14 hours after transfection, cell lysates were analysed by western blot with anti-Flag antibody. (B) TRIP6 RNAi inhibits expression of endogenous TRIP6. 293 cells were transfected with pSuper or pSuper-TRIP6. Transfected cells were selected with puromycin (10 μg ml^{-1}) for 1 week. Cell lysates were analysed by western blot with a rabbit anti-TRIP6 and a monoclonal anti-β-actin antibody. (C) Effects of TRIP6 RNAi on NF-κB activation by various stimuli. NF-κB reporter gene assays with the cell lines stably transfected with TRIP6 RNAi or control vector were performed as in Figs 4, 6.

that TRIP6 is involved in NF-κB activation by these stimuli under physiological conditions.

In these experiments, TRIP6 RNAi could only partially inhibit TNF- and IL-1-induced NF-κB activation (Fig. 7C). The partial inhibition might be explained by the fact that the RNAi could not completely knock out TRIP6 expression. Also, TRIP6 might potentiate NF-κB activation but not be required for NF-κB activation by the various stimuli.

TRIP6 is involved in RIP2- and Nod1-mediated ERK activation

Previously, it has been shown that RIP2 is also involved in ERK activation (McCarthy et al., 1998; Inohara et al., 1998; Thome et al., 1998). We determined whether TRIP6 is involved in RIP2-mediated ERK activation by Elk1 (a transcription factor activated by ERK) reporter-gene assays. In these experiments, we found that overexpression of TRIP6 alone had minimal effects on Elk1 activation (Fig. 8A). Interestingly, TRIP6-(280-480), a TRIP6 deletion mutant that only contains the C-terminal LIM domains of TRIP6, could strongly activate Elk1 (Fig. 8A). In these experiments, the N-terminal domain of TRIP6 had no significant effects (Fig. 8A). These data suggest that the N-terminal domain of TRIP6 has an inhibitory effect on the ability of TRIP6 to activate Elk1.

Consistent with a role in RIP2-mediated ERK activation, TRIP6 potentiated RIP2- and Nod1-mediated Elk1 activation in a dose-dependent manner in reporter-gene assays (Fig. 8B). These data suggest that TRIP6 is involved in both RIP2-mediated NF-κB and ERK activation pathways.

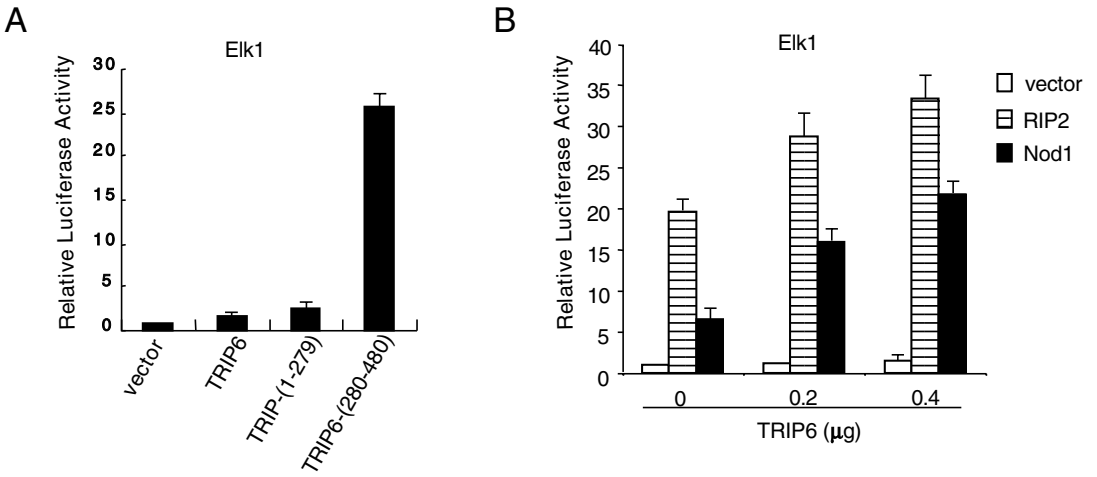

Fig. 8. TRIP6 potentiates RIP2- and Nod1-mediated Elk1 activation. (A) Effects of TRIP6 and its mutants on Elk1 activation. The detection of ERK activity was measured by Elk1 activity according to the PathDetect in vivo signal transduction pathway trans reporting systems protocols (Stratagene, La Jolla, CA). 293 cells (2×10^5) were transfected with 0.5 μg pFR plasmid, 0.05 μg the trans-activating plasmid pFA-Elk1, 0.2 μg Rous sarcoma virus (RSV)/β-galactosidase plasmid (as transfection control) and 0.2 μg plasmid encoding the indicated TRIP6 or its mutant. Luciferase and β-galactosidase reporter assays were performed 16 hours after transfection. Luciferase activities were normalized on the basis of β-galactosidase activities. (B) TRIP6 potentiates RIP2- and Nod1-mediated Elk1 activation. 293 cells (2×10^5) were transfected with 0.5 μg pFR plasmid, 0.05 μg the trans-activating plasmid pFA-Elk1, 0.2 μg RSV/β-galactosidase plasmid (as transfection control), 0.2 μg RIP2 or Nod1 plasmid and the indicated amount of TRIP6 expression plasmid. Luciferase and β-galactosidase reporter assays were performed 16 hours after transfection. Luciferase activities were normalized on the basis of β-galactosidase activities.

Discussion

Previous studies demonstrated that RIP2 is an essential component of multiple NF-κB activation pathways and is crucially involved in inflammation, innate and adaptive immune responses. The mechanisms responsible for the involvement of RIP2 in multiple pathways are not well understood. In this report, we have identified TRIP6 as a RIP2-interacting protein and demonstrated that TRIP6 is involved in NF-κB activation by TNF, IL-1, TLR2 and Nod1.

TRIP6 is a member of a subfamily of LIM-domain-containing proteins that also includes zyxin, lipoma preferred partner (LPP), Ajuba and Hic-5 (Wang and Gilmore, 2003; Beckerle, 1997). Most proteins of this family primarily reside in focal adhesion plaques in resting cells but, under a range of circumstances, several can shuttle between the cytoplasm and the nucleus (Wang and Gilmore, 2003; Beckerle, 1997). TRIP6 was originally identified in yeast two-hybrid screens as a protein that interacts with the thyroid-hormone receptor in a hormone-dependent manner (Lee et al., 1995). It has also been shown that TRIP6 is associated with the tyrosine phosphatase PTP1E/PTP-BL (Murthy et al., 1999), the OpaP protein of *Neisseria gonorrhoeae* (Williams et al., 1998), the adaptor protein RIL (Cuppen et al., 2000), the v-Rel oncoprotein (Zhao et al., 1999), the integrin-associated adaptor protein $p130^{Cas}$ (Yi et al., 2002) and the lysophosphatidic-acid receptor (Xu et al., 2004). TRIP6 contains three LIM domains at its C-terminus and interacts with its binding partners through its LIM domains. TRIP6 also contains a NES at its N-terminus and it has been shown that TRIP6 shuttles between cytoplasmic and nuclear compartments (Wang and Gilmore, 2001).

Although multiple interacting partners have been identified for TRIP6, its physiological functions are not well defined. It has been suggested that TRIP6 is involved in cell migration and transmits signals between adhesion plaques and the nucleus (Wang and Gilmore, 2001; Wang and Gilmore, 2003; Beckerle, 1997). Because TRIP6 interacts with two transcription factors (the thyroid-hormone receptor and v-Rel) and activates transcription when fused to a heterologous DNA-binding domain, it has been proposed that TRIP6 is a transcriptional co-activator (Zhao et al., 1999). Because the human *TRIP6* gene is located at chromosome 7q22, a region that is rearranged or deleted in several human tumors, it has also been proposed that TRIP6 might have a role in oncogenesis (Yi and Beckerle, 1998).

In this study, we identified TRIP6 as a RIP2-interacting protein. The LIM domains of TRIP6 are responsible for its interaction with RIP2. In untransfected cells, TRIP6 and RIP2 interact in a TNF- or IL-1-dependent manner, suggesting that the interaction between TRIP6 and RIP2 is stimulation-dependent. TRIP6 interacts with TRAF2, a protein crucially involved in TNF signaling. Overexpression of TRIP6 potentiates TNF-induced NF-κB activation, whereas a dominant-negative mutant or RNAi of TRIP6 significantly inhibits TNF-induced NF-κB activation. Taken together, these data suggest that TRIP6 is a physiological component of TNF-induced NF-κB activation pathway.

Previously, gene knockout studies had indicated that RIP2 is not required for TNF-induced IκBα phosphorylation and IL-6 production (Kobayashi et al., 2002; Ruefli-Brasse et al., 2004), but these studies cannot exclude the possibility that RIP2 is indeed involved in TNF signaling and that RIP2 deficiency is complemented by other RIP family members, such as RIP. Although we identified TRIP6 as a RIP2-interacting protein using yeast two-hybrid screens, co-immunoprecipitation experiments suggest that TRIP6 interacts with multiple signaling proteins involved in TNF, IL1, TLR and Nod1-induced NF-κB activation, including TRAF2, TRAF6, MyD88, Tollip and IRAK1 (Figs 3, 5). Thus, the involvement of TRIP6 in the distinct NF-κB activation pathways might be mediated by interacting with different signaling proteins.

In addition to its role in TNF signaling, TRIP6 also interacts with IL-1 receptors, TLR2 and Nod1. Overexpression of TRIP6 potentiates NF-κB activation by IL-1, TLR2 and Nod1, whereas a dominant-negative mutant or RNAi of TRIP6 inhibits NF-κB activation by IL-1, TLR2 and Nod1, suggesting that TRIP6 is involved in NF-κB activation by IL-1, TLR2 and Nod1.

Taken together, our data have established a physical and functional association between TRIP6 and RIP2. Our findings point to the possibility that the involvement of RIP2 in multiple NF-κB activation pathways is mediated through TRIP6.

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