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### **Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN- $\beta$ (TRIF) Associates with TNF Receptor-Associated Factor 6 and TANK-Binding Kinase 1, and Activates Two Distinct Transcription Factors, NF- $\kappa$ B and IFN-Regulatory Factor-3, in the Toll-Like Receptor Signaling**

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# Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN- $\beta$ (TRIF) Associates with TNF Receptor-Associated Factor 6 and TANK-Binding Kinase 1, and Activates Two Distinct Transcription Factors, NF- $\kappa$ B and IFN-Regulatory Factor-3, in the Toll-Like Receptor Signaling<sup>1</sup>

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We previously reported a new Toll/IL-1R (TIR)-containing molecule, named TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF). Although initial study indicated that TRIF possesses the ability to activate not only the NF- $\kappa$ B-dependent but also the IFN- $\beta$  promoters, the molecular mechanisms of TRIF-induced signaling are poorly understood. In this study, we investigated the signaling cascades through TRIF. TNF receptor-associated factor (TRAF)6 interacted with TRIF through the TRAF domain of TRAF6 and TRAF6-binding motifs found in the N-terminal portion of TRIF. Disruption of TRAF6-binding motifs of TRIF disabled it from associating with TRAF6, and resulted in a reduction in the TRIF-induced activation of the NF- $\kappa$ B-dependent but not IFN- $\beta$  promoter. TANK-binding kinase (TBK)-1, which was recently reported to be a kinase of IFN regulatory factor-3, which is an essential transcription factor for IFN- $\beta$  expression, also associated with the N-terminal region of TRIF. Moreover, the association between TRIF and TBK1 appeared to require the kinase activity of TBK1, as well as phosphorylation of TRIF. Because TRAF6 and TBK1 bind close the region of TRIF, it seems that TRAF6 physically prevents the association between TRIF and TBK1. Taken together, these results demonstrate that TRIF associates with TRAF6 and TBK1 independently, and activates two distinct transcription factors, NF- $\kappa$ B and IFN regulatory factor-3, respectively. *The Journal of Immunology*, 2003, 171: 4304–4310.

Innate immunity against invading pathogens relies on sensing specific molecular features expressed in microorganisms by pattern recognition receptors. This recognition is mediated by a set of germline-encoded receptors called Toll-like receptors (TLRs)<sup>3</sup> (1–3). So far, 10 members of the human TLR family have been discovered. For example, TLR4 and TLR3 recognize LPS from Gram-negative bacteria and dsRNA, which is produced by viral infection, respectively. The recognition of these structures by individual TLRs triggers the activation of a common intracellular signaling pathway leading to the nuclear translocation of a transcription factor, NF- $\kappa$ B. This signaling eventually culminates in the production of proinflammatory cytokines to engage host de-

fense responses and bridge acquired immunity governed by T and B lymphocytes (2).

The cytoplasmic region of TLRs shares a stretch of protein module called the Toll/IL-1R (TIR) domain, which mediates homophilic and heterophilic interactions between TLRs and TIR-containing adaptors (2). Myeloid differentiation factor 88 (MyD88) is an adaptor protein that harbors a TIR domain as well as a death domain. MyD88 is recruited to the receptor complex through TIR domains when TLRs recognize their ligands. The IL-1R-associated kinase (IRAK) family, a series of death domain-containing serine/threonine kinases, is also recruited to the receptor complex, and then activated. Sequential activation of IRAKs results in the activation of TNFR-associated factor 6 (TRAF6) and finally NF- $\kappa$ B (2).

Through studies with MyD88-deficient mice and cells, it has been shown that MyD88 plays a pivotal role and is essential for production of inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in response to ligands for individual TLRs (2, 4, 5). However, in addition to this pathway, studies with MyD88-deficient mice also revealed that individual TLRs can induce alternative and specific effector responses to distinct microbial pathogens (2, 5). Notably, stimulation with TLR4 and TLR3 ligands, LPS and dsRNA, can induce IFN- $\beta$  expression and subsequent induction of a set of IFN-inducible genes independently of MyD88, indicating that another adaptor protein may play a critical role in inducing IFN- $\beta$  expression (5, 6). We previously reported that IFN regulatory factor (IRF)-3, a transcriptional factor responsible for induction of the IFN- $\beta$  gene, is activated in response to LPS in a MyD88-independent manner, and involved in the LPS-induced MyD88-independent pathway (7). IRF-3 is also known to be activated by TLR3 ligand (8).

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<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; TIR, Toll/IL-1R; IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor; MyD88, myeloid differentiation factor 88; IRF, IFN regulatory factor; 6; TRIF, TIR domain-containing adaptor inducing IFN- $\beta$ ; TBK, TANK-binding kinase; ELAM, endothelial cell-leukocyte adhesion molecule; TIRAP, TIR domain-containing adaptor protein; IKK, I $\kappa$ B kinase.

Recently, we have identified a new TIR-containing adaptor molecule termed TIR domain-containing adaptor inducing IFN- $\beta$  (TRIF) (9). Initial study showed that TRIF possesses the ability to activate not only the NF- $\kappa$ B but also the IFN- $\beta$  promoter, and associates with both TLR3 (possibly TLR4) and IRF-3 (9). The truncated mutant TRIF $\Delta$ C, which encompasses the N-terminal half and TIR domain of TRIF, still possesses the ability to activate both the NF- $\kappa$ B and IFN- $\beta$  promoter. Overexpression of the truncated mutant TRIF $\Delta$ N, which encompasses the TIR domain and C-terminal half, also activates the NF- $\kappa$ B-dependent promoter whereas it fails to activate the IFN- $\beta$  promoter. These observations suggest that the N-terminal portion of TRIF bears a dual role to activate the NF- $\kappa$ B and IFN- $\beta$  promoter. However, the molecular mechanism behind the TRIF-induced activation of the NF- $\kappa$ B and IFN- $\beta$  promoter is poorly understood.

In this paper, we attempted to define the downstream cascades of TRIF through identification of TRIF-associated molecules, and showed that TRIF associates with two distinct proteins, TRAF6 and TANK-binding kinase (TBK) 1. TRAF6 binds TRIF through TRAF6-binding motifs found in the N-terminal region of TRIF, and this association appears to be necessary for activation of NF- $\kappa$ B, while TBK1 mediates the induction of IFN- $\beta$  expression. Thus, TRIF acts as a scaffold to assemble signaling proteins TRAF6 and TBK1, regulating two distinct pathways leading to the activation of the NF- $\kappa$ B and IFN- $\beta$  promoter, respectively.

## Materials and Methods

### Cell culture and reagents

Human embryonic kidney 293 cells and mouse peritoneal macrophages were cultured in DMEM and RPMI 1640 medium supplemented with 10% FCS, respectively. The generation of 293 cells stably expressing human TLR3 was described previously (9), and two independent clones were used for experiments. Peritoneal macrophages were isolated from C57BL/6J mice (SLC, Shizuoka, Japan). Briefly, mice were injected i.p. with 2 ml of 4% thioglycollate (Sigma-Aldrich, St. Louis, MO). After 3 days, peritoneal exudate cells were isolated by washing the peritoneal cavity with ice-cold HBSS. These cells were incubated for 2 h, and adherent cells were used as peritoneal macrophages.

LPS from *Salmonella minnesota* Re 595 was obtained from Sigma-Aldrich. Anti-TRAF6 polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TRIF Ab was raised against corresponding amino acids 672–684 or 718–732 of mouse TRIF for immunoprecipitation or immunoblotting, respectively.

### Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the Matchmaker two-hybrid system (Clontech Laboratories, Palo Alto, CA). To construct a bait plasmid, the N-terminal region of human TRIF (TRIF $\Delta$ TIR $\Delta$ C; aa 1–391) was cloned into pAS2-1 downstream of the Gal4 DNA-binding domain (BD-TRIF $\Delta$ TIR $\Delta$ C). Yeast strain Y190 cells were cotransformed with BD-TRIF $\Delta$ TIR $\Delta$ C and the human lung MATCHMAKER cDNA library (Clontech Laboratories). From the screening of  $\sim 1 \times 10^6$  clones, several positive clones were obtained. Positive clones were picked, and the pACT2 library plasmids were recovered from individual clones and expanded in *Escherichia coli*. The insert cDNA was sequenced, and then characterized using the BLAST program (National Center for Biotechnology Information, Bethesda, MD).

### Plasmids construction

To construct a mammalian expression plasmid for Myc-tagged human TRAF6-CT, an *EcoRI-XhoI* fragment was prepared from a positive pACT2 clone, and then inserted into the vector pCMV-Myc (Clontech Laboratories). Expression plasmids for the wild-type and  $\Delta$ C mutant of human TRIF, and the wild-type and K38A mutant of human I $\kappa$ B kinase *i* (IKK $\alpha$ ) were described previously (9, 10). The cDNA fragments encoded by other expression proteins used in this paper were amplified by PCR from a human spleen cDNA library (Clontech Laboratories), digested with appropriate restriction enzymes, and inserted into pFLAG-CMV2 (Sigma-Aldrich), pCMV-Myc or pEF-BOS for the N-terminal FLAG-tagged and Myc-tagged proteins. To generate mutant TRIF for E88A, E88/252A and E88/252/303E (3A), and a kinase negative mutant of human TBK1, site-

directed mutagenesis using QuickChange XL-Site Directed Mutagenesis Kit was performed as specified by the manufacturer (Stratagene, La Jolla, CA). The sequences of DNA fragments obtained by PCR were confirmed by DNA sequencing. The sequence of each primer will be provided upon request.

### Transfection, immunoprecipitation, and immunoblot analysis

Two million 293 cells were seeded on a 60-mm-diameter dish. Twenty-four hours later, cells were transiently transfected with a total of 5.0  $\mu$ g of empty or the indicated plasmids using LIPOFECTAMINE 2000 as specified by the manufacturer (Invitrogen, Carlsbad, CA). For immunoprecipitation, cells were harvested 36 h after transfection and lysed in lysis buffer containing 1.0% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0 mM EDTA, 10 mM  $\beta$ -glycerophosphate, 1.0 mM Na $_3$ VO $_4$ , and a protease inhibitor mixture (Complete; Roche Diagnostics, Indianapolis, IN). Cell lysates were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h and then incubated with protein G-Sepharose together with either 1.0  $\mu$ g of anti-FLAG M2 mAb (Sigma-Aldrich) or 1.0  $\mu$ g of anti-Myc 9B11 mAb (Cell Signaling Technology, Beverly, MA) for 12 h by rotation. The beads were washed four times with lysis buffer. The immunoprecipitants were eluted by boiling with Laemmli sample buffer, separated on a 4–20% polyacrylamide gradient gel, and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with HRP-conjugated anti-FLAG mAb (Sigma-Aldrich) or anti-Myc 9E10 mAb (Santa Cruz Biotechnology) for 1 h. After a wash with TBS-T (25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20), peroxidase activity was detected with the ECL system (PerkinElmer Life Sciences, Boston, MA).

### Luciferase reporter assay

293 cells that had been seeded on 24-well plate at a density of  $2 \times 10^5$  per well were transiently transfected with 100 ng of either the endothelial cell leukocyte adhesion molecule (ELAM)-1 promoter-derived NF- $\kappa$ B luciferase reporter plasmid, which was kindly provided by Dr. D. T. Golenbock (University of Massachusetts Medical School, Worcester, MA), or murine IFN- $\beta$  promoter reporter plasmid (11), together with a total of 1.0  $\mu$ g of the indicated expression vectors. Forty-eight hours later, the luciferase activity in the total cell lysate was measured using the Dual-luciferase reporter assay system (Promega, Madison, WI). The *Renilla*-luciferase reporter gene (50 ng) was used as an internal control.

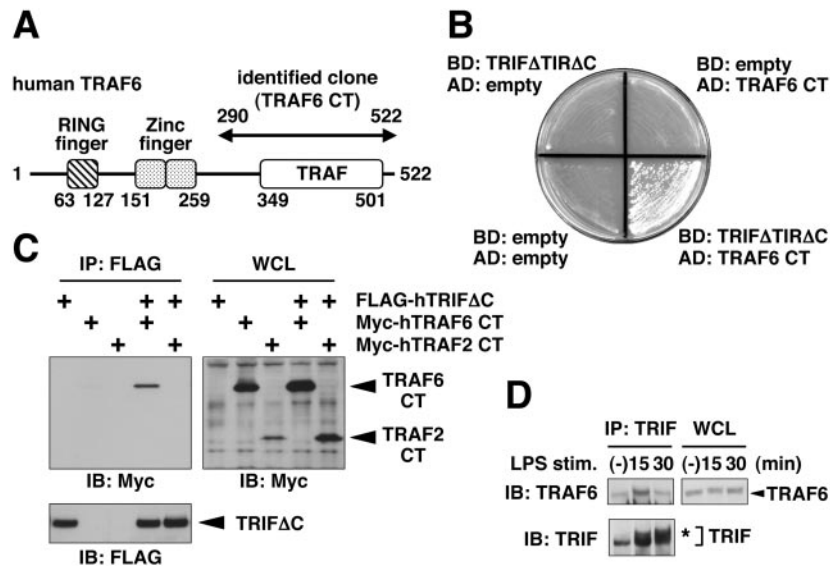
## Results

### Identification of TRIF-interacting molecules

To investigate the TRIF-mediated signaling cascades, we performed a yeast two-hybrid screening. We used as bait the N-terminal portion of human TRIF (TRIF $\Delta$ TIR $\Delta$ C) because overexpression of TRIF $\Delta$ C induced activation of both the NF- $\kappa$ B-dependent and IFN- $\beta$  promoters (9). Through the screening of a human lung cDNA library, several clones were isolated as positive. One of them harbored the whole TRAF domain of TRAF6 (Fig. 1, A and B).

The association of TRIF with the C-terminal half of TRAF6 (TRAF6 CT) was initially confirmed by coimmunoprecipitation assay in mammalian cells. 293 cells were transiently expressed with Myc-tagged TRAF6 CT or TRAF2 CT, another member of the TRAF family, together with FLAG-tagged full-length TRIF. Cell lysates were immunoprecipitated with anti-FLAG mAb, and then immunoblotted with anti-Myc mAb to detect TRIF-associated Myc-tagged protein. As shown in Fig. 1C, when TRAF6 CT was cotransfected with TRIF, their association was observed. In contrast, TRAF2 CT was not detected in TRIF immunoprecipitants.

The interaction of endogenous TRIF and TRAF6 was also examined by coimmunoprecipitation experiments, using mouse peritoneal macrophages and Abs specific for TRIF and TRAF6. Before stimulation with LPS, TRAF6 was coimmunoprecipitated with TRIF (Fig. 1D). After 15 min stimulation with LPS, the interaction of TRIF and TRAF6 was enhanced. Interestingly, TRIF was modified and slowly migrated in response to LPS (Fig. 1D, \*). These observations indicated that TRIF binds the TRAF domain of TRAF6.



**FIGURE 1.** TRIF and TRAF6 associate in yeast and mammalian cells. *A*, Schematic structure of human TRAF6. A yeast clone expressing the C-terminal region of TRAF6 (arrow; TRAF6 CT) was obtained as positive to interact with the N-terminal portion of TRIF (TRIF $\Delta$ TRIF $\Delta$ C) by yeast two hybrid screening. *B*, Association between TRIF and TRAF6 in yeast. Yeast cells were cotransformed with the indicated combinations of BD- and AD-plasmids, and grown on a synthetic dextrose agar plate lacking leucine, tryptophan, and histidine to observe the indicative interactions. *C*, TRIF-TRAF6 interaction in mammalian cells. 293 cells ( $2 \times 10^6$ ) were transiently transfected with 1.5  $\mu$ g of Myc-TRAF6 CT, 1.5  $\mu$ g of Myc-TRAF2 CT, and 3.5  $\mu$ g of FLAG-TRIF $\Delta$ C expression vectors. The total amount of plasmid DNA was kept at 5.0  $\mu$ g by using an empty vector. Thirty-six hours after transfection, cells were lysed, immunoprecipitated with anti-FLAG mAb (IP), and then immunoblotted with anti-FLAG or anti-Myc mAb (IB). *D*, Endogenous association between TRIF and TRAF6. Mouse peritoneal macrophages were stimulated with 1.0  $\mu$ g/ml LPS for the indicated periods. Cell lysates were prepared, immunoprecipitated with anti-TRIF Ab, and then immunoblotted with anti-TRAF6 and anti-TRIF Abs to determine the association of TRIF and TRAF6 (*left panels*). Expression of TRAF6 was also determined by Western blot analysis using an aliquot of the same lysates (*right panel*).

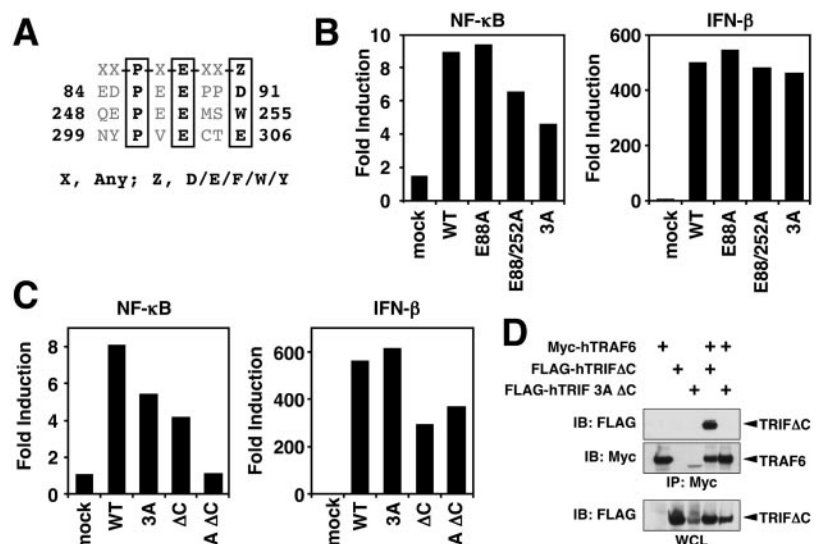
#### TRAF6-binding motifs found in TRIF are required for NF- $\kappa$ B activation

TRAF6 is known as an intracellular signaling molecule that activates NF- $\kappa$ B in the TLR-signaling pathway (2). The TRAF domain of TRAF6 can reportedly preferably recognize a motif (P-x-E-x-x-acidic or aromatic residue) for interactions with other signaling molecules (12). For example, IRAK-1, which is known to associate with the TRAF domain of TRAF6, has three TRAF6-binding motifs, and the NF- $\kappa$ B-dependent reporter activation induced by IRAK-1 was severely reduced by the substitution of P<sub>0</sub>-residues

(from glutamic acid to alanine) (12). Interestingly, both human and mouse TRIF also contain three TRAF6-binding motifs, and all of them are located in the N-terminal region (Fig. 2A).

To test the functional importance of TRAF6-binding motifs of TRIF, we generated a series of mutants by substituting glutamic acids at 88, 88/252, and 88/252/303 of human TRIF with alanines, and compared their abilities to activate NF- $\kappa$ B-dependent and IFN- $\beta$  promoters by luciferase reporter assay. As shown in Fig. 2B, the E88A mutant strongly activated NF- $\kappa$ B as well as wild-type TRIF. Activation of NF- $\kappa$ B by double (E88/252A) and triple (E88/252/

**FIGURE 2.** TRAF6-binding motifs in TRIF mediates activation of the NF- $\kappa$ B-dependent, but not the IFN- $\beta$ , promoter. *A*, Scheme of TRAF6-binding motifs found in human TRIF. *B* and *C*, Functional analysis for the interaction of TRIF and TRAF6. 293 cells were transiently transfected with empty vector (mock) or expression vector for wild-type TRIF (WT), TRIF harboring single (E88A), double (E88/252A), or triple (3A) mutations in the TRAF6-binding motifs, C-terminal truncated TRIF ( $\Delta$ C), and a 3A mutant of TRIF $\Delta$ C (3A $\Delta$ C), together with the NF- $\kappa$ B-dependent (*left panel*) or the IFN- $\beta$  promoter luciferase reporter (*right panel*). Thirty-six hours after transfection, luciferase activity was measured. The data are representative of three independent experiments. *D*, Substitutions of TRAF6-binding motifs in TRIF completely abolished their association. 293 cells ( $2 \times 10^6$ ) were transiently cotransfected with 2.5  $\mu$ g of the indicated combinations of plasmids. The total amount of plasmid DNA was kept at 5.0  $\mu$ g by using an empty vector. Thirty-six hours after transfection, cells were lysed, immunoprecipitated with anti-Myc mAb (IP), and then immunoblotted with anti-FLAG or anti-Myc mAb (IB).

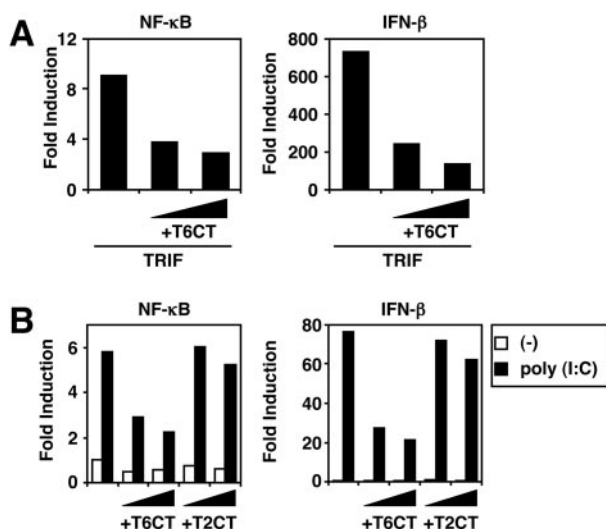


303A; 3A) mutants was partially reduced, compared with the wild-type and E88A mutant. In contrast, all mutants activated the IFN- $\beta$  promoter at the same level as the wild-type TRIF.

The remaining activation of NF- $\kappa$ B induced by the TRIF 3A mutant might be mediated by the C-terminal portion of TRIF because TRIF possessed the ability to activate the NF- $\kappa$ B-dependent reporter even with its N-terminal region truncated (9). In this regard, we further constructed a 3A mutant of TRIF lacking the C-terminal region (TRIF 3A $\Delta$ C), and investigated its ability to activate the NF- $\kappa$ B-dependent promoter. As shown in Fig. 2C, the TRIF 3A $\Delta$ C mutant completely lost the ability to activate the NF- $\kappa$ B-dependent promoter but not the IFN- $\beta$  promoter, as determined by reporter assay. Moreover, the 3A $\Delta$ C mutant completely lost the ability to associate with TRAF6 in the mammalian cells (Fig. 2D). Collectively, these observations suggested that the N-terminal region of TRIF directly associates with the TRAF domain of TRAF6, and this association is necessary and sufficient for activation of the NF- $\kappa$ B-dependent but not IFN- $\beta$  promoter. Furthermore, these results indicated that TRIF might have at least two pathways for NF- $\kappa$ B activation. One is mediated by the TRIF N-terminal portion via TRAF6, and the other is induced from its C-terminal region.

*TRAF domain of TRAF6 acts as a dominant-negative mutant to prevent TRIF-induced activation of the NF- $\kappa$ B-dependent and IFN- $\beta$  promoters*

We next investigated whether TRAF6 CT acts as a dominant-negative mutant for TRIF-induced activation of the NF- $\kappa$ B-dependent and IFN- $\beta$  promoters. Our previous report indicated that ectopic expression of TRIF significantly induced activation of both the NF- $\kappa$ B-dependent and the IFN- $\beta$  promoters (Ref. 9 and Fig. 3A).



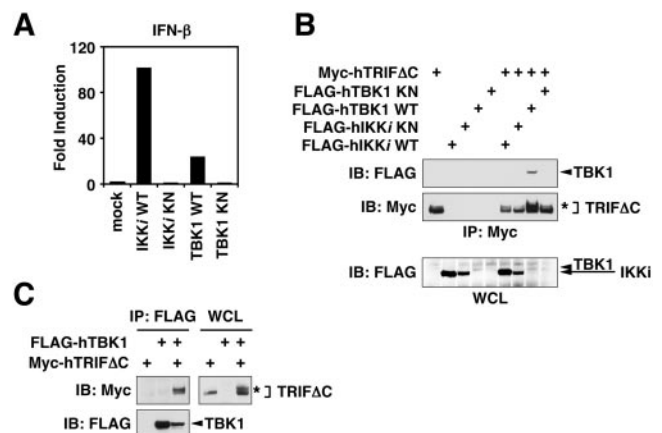
**FIGURE 3.** TRAF domain of TRAF6 acts as a dominant-negative inhibitor of TRIF- and TLR3-induced activation of the NF- $\kappa$ B-dependent and IFN- $\beta$  promoters. *A*, 293 cells were transfected with 500 ng of the TRIF-expression vector and the NF- $\kappa$ B (*left panel*) or IFN- $\beta$  (*right panel*) reporter, together with the expression vector for the TRAF domain of TRAF6 (T6CT; 100 or 250 ng). After 36 h, relative NF- $\kappa$ B-dependent or IFN- $\beta$  promoter activity was measured by the luciferase assay. *B*, 293 cells stably expressing TLR3 were transfected with the expression vector for the T6CT (100 or 250 ng) or TRAF domain of TRAF2 (T2CT; 100 or 250 ng), together with the NF- $\kappa$ B (*left panel*) or IFN- $\beta$  (*right panel*) reporter. Cells were left unstimulated ( $\square$ ) or stimulated with 50  $\mu$ g/ml poly (I:C) for 9 h ( $\blacksquare$ ), and luciferase activity was measured. Two independently isolated 293 clones that express human TLR3 were used for the experiments, and similar results were obtained.

When TRAF6 CT was coexpressed with TRIF in 293 cells, TRIF-induced NF- $\kappa$ B activation was inhibited in a dose-dependent manner. Surprisingly, the activation of the IFN- $\beta$  promoter by TRIF was also inhibited by TRAF6 CT.

TRIF was speculated to be involved in TLR3 signaling, and overexpression of TLR3 in 293 cells enabled activation of both the NF- $\kappa$ B-dependent reporter and IFN- $\beta$  promoter in response to poly(I:C) (9). Moreover, it was shown that the TLR3-mediated activation of NF- $\kappa$ B depends on TRAF6 but not IRAK-1 (13). Because TRIF associates with both TRAF6 and TLR3, it is plausible that TRIF is an adaptor that links TLR3 with TRAF6. Therefore, we next investigated whether TRAF6 CT could block TLR3-signaling. 293 cells stably expressing human TLR3 were transfected with the expression vector for TRAF6 CT or TRAF2 CT, and then left unstimulated or stimulated with poly(I:C). As shown in Fig. 3B, NF- $\kappa$ B-responsive promoter activation in response to poly(I:C) was specifically inhibited by expression of TRAF6 CT but not TRAF2 CT. Expression of TRAF6 CT also inhibited IFN- $\beta$  promoter activation in response to poly(I:C). These observations suggested that TRAF6, but not TRAF2, was also involved in TLR3-dependent signaling pathways.

*TBK1, but not IKKi, associates with the N-terminal portion of TRIF, and activates the IFN- $\beta$  promoter*

Although TRAF6 CT inhibited TRIF-induced NF- $\kappa$ B-responsive and IFN- $\beta$  promoter activation, substitution of the TRAF6-binding motifs of TRIF affected the activation of the NF- $\kappa$ B-dependent promoter, but not the IFN- $\beta$  promoter, suggesting that other molecule(s) might be involved in the activation of the IFN- $\beta$  promoter directly downstream of TRIF. Recently, it has been reported that IKKi (also known as IKK $\epsilon$ ) and TBK1 (also known as NF- $\kappa$ B-activating kinase and T2K), which were originally shown to activate NF- $\kappa$ B, are responsible for the activation of the IFN- $\beta$  promoter by acting as IRF-3 kinases (14, 15). Consistent with previous reports, the ectopic expression of these kinases strongly activated the IFN- $\beta$  promoter (Fig. 4A). Moreover, it was shown



**FIGURE 4.** TRIF associates with TBK1 but not IKKi. *A*, Overexpression of the wild-type (WT), but not kinase-negative form (KN), of IKKi and TBK1 induces activation of the IFN- $\beta$  promoter. 293 cells were transfected with 500 ng of the indicated expression plasmid together with the IFN- $\beta$  reporter plasmid. After 36 h, relative IFN- $\beta$  promoter activity was measured by the luciferase assay. *B* and *C*, Interaction between TRIF and TBK1. 293 cells ( $2 \times 10^6$ ) were transiently cotransfected with 2.5  $\mu$ g of the indicated combinations of plasmids. The total amount of plasmid DNA was kept at 5.0  $\mu$ g by using an empty vector. Thirty-six hours after transfection, whole cell lysates were prepared, immunoprecipitated with anti-Myc (*B*) or anti-FLAG mAb (*C*) (IP), and then immunoblotted with the indicated Abs (IB).

that the N-terminal portion of TRIF activated the IFN- $\beta$  promoter, and endogenous IRF-3 was coimmunoprecipitated with the overexpressed TRIF (9). Therefore, we next investigated whether or not TRIF $\Delta$ C associated with IKK $\alpha$  and TBK1. 293 cells were transfected with expression vectors for FLAG-tagged wild-type or kinase-negative forms of IKK $\alpha$  or TBK1, together with Myc-tagged TRIF $\Delta$ C, and then TRIF $\Delta$ C was immunoprecipitated using anti-Myc mAb. As shown in Fig. 4B, wild-type TBK1 was coimmunoprecipitated with anti-Myc mAb. In contrast to previous reports showing that IKK $\alpha$  was coimmunoprecipitated with TRIF (15), interaction between TRIF and IKK $\alpha$  (both the wild-type and kinase-negative mutant) was not observed under the conditions. Interestingly, when wild-type TBK1 was cotransfected with TRIF $\Delta$ C, a slowly migrating form of TRIF $\Delta$ C was observed, as was the case for interaction between endogenous TRIF and TRAF6 (Fig. 1D and 4B, \*). Furthermore, this slow migrating band was also detected when wild-type IKK $\alpha$  was cotransfected with TRIF $\Delta$ C, albeit at a lower level than with TBK1 coexpression. Treatment of the lysate with phosphatase abolished this slow migrating band, indicating that the slow migration of TRIF was due to phosphorylation (data not shown).

We next coexpressed FLAG-tagged TBK1 and Myc-tagged TRIF $\Delta$ C in 293 cells, and immunoprecipitation was performed using anti-FLAG mAb. As shown in Fig. 4C, the ratio of the slow migrating band of TRIF was significantly increased after immunoprecipitation compared with that of whole cell lysates, indicating that phosphorylated TRIF $\Delta$ C was immunoprecipitated more efficiently than nonphosphorylated TRIF $\Delta$ C with TBK1. This result suggested that phosphorylated TRIF forms a complex with TBK1 with a higher affinity than unphosphorylated TRIF.

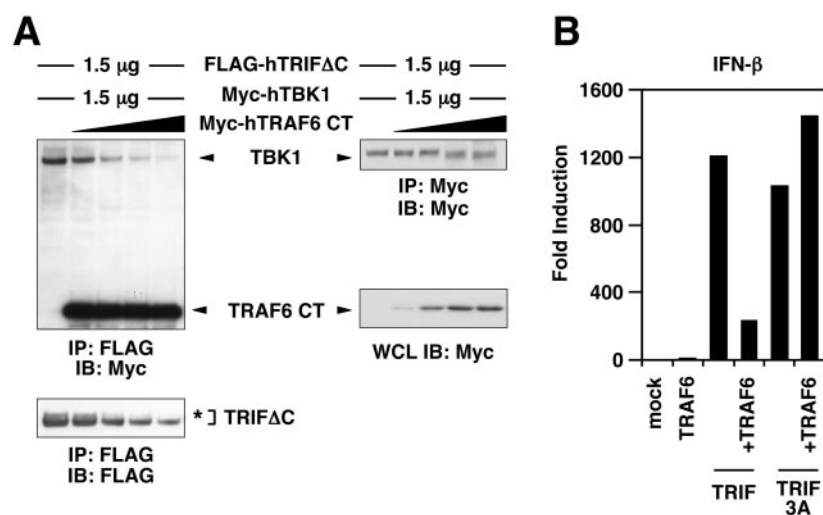
#### TRAF6 and TBK1 associate with TRIF via different regions

Next, we investigated whether TRAF6 CT and TBK1 competed with each other in the association with TRIF. For these experiments, 293 cells were transfected with FLAG-tagged TRIF $\Delta$ C and Myc-tagged TBK1 expression vectors, together with different amounts of Myc-tagged TRAF6 CT, and then TRIF $\Delta$ C was im-

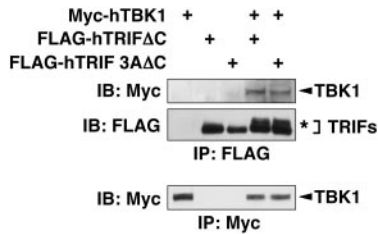
muno-precipitated with anti-FLAG mAb. As shown in Fig. 5A, although both TBK1 and TRAF6 CT were coimmunoprecipitated with TRIF $\Delta$ C, increased amounts of TRAF6 CT repressed the association between TRIF $\Delta$ C and TBK1. This observation suggested that TRAF6 CT acts as a competitor, rather than a dominant-negative form, for TRIF/TBK1-induced IFN- $\beta$  promoter activation. It is possible that the full length of TRAF6 also might act as a competitor for TRIF-induced IFN- $\beta$  promoter activation. In this regard, coexpression of the full length of TRAF6 inhibited TRIF-induced IFN- $\beta$  promoter activation (Fig. 5B). In contrast, the activation of the IFN- $\beta$  promoter activation induced by the TRIF 3A mutant was not affected by coexpression of TRAF6. Furthermore, we examined whether TBK1 used the TRAF6-binding motifs of TRIF for interaction. As shown in Fig. 6, TBK1 was coimmunoprecipitated with both TRIF $\Delta$ C and TRIF 3A $\Delta$ C. These observations suggested that TRAF6 and TBK1 associated with TRIF in a similar but not the same region, and that either the full length or the TRAF domain of TRAF6 acted as a competitive antagonist in the TRIF-TBK1 association as well as TRIF-induced IFN- $\beta$  promoter activation.

#### Discussion

Previously, we and other groups independently identified a new TIR-containing adaptor molecule, termed TRIF (9, 16). It has been suggested that TRIF is involved in TLR3-mediated signaling because it can associate with TLR3, and differs from other TIR-containing adaptors, such as MyD88 and TIR domain-containing adaptor protein (TIRAP), in that it has the ability to activate the IFN- $\beta$  promoter. Analysis of MyD88-deficient mice revealed the presence of MyD88-independent pathways leading to the activation of NF- $\kappa$ B and IRF-3, which ultimately culminates in induction of the expression of IFN-inducible genes and the maturation of dendritic cells in response to LPS or poly(I:C) (6, 7, 17). Although TIRAP, an additional TIR-containing adaptor protein, was suggested to be involved in the MyD88-independent pathway, both the expression of IFN-inducible genes and maturation of dendritic



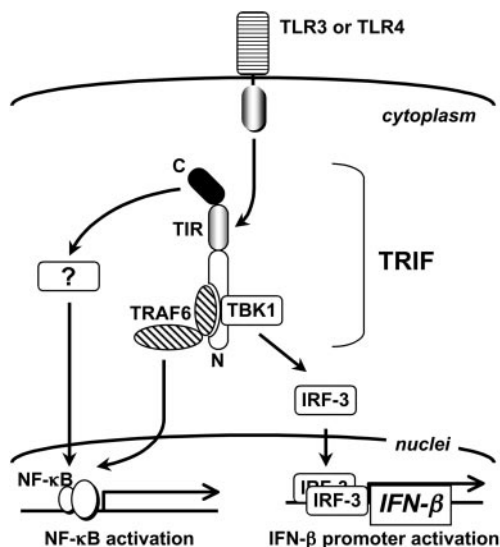
**FIGURE 5.** TRAF6 acts as a competitor for the association of TRIF with TBK1. *A*, TRAF6 CT inhibits the association between TRIF and TBK1. 293 cells ( $2 \times 10^6$ ) were transiently transfected with 1.5  $\mu$ g of FLAG-tagged TRIF $\Delta$ C and Myc-tagged TBK1, together with 2.0  $\mu$ g of empty vector or 0.1, 0.5, 1.0, and 2.0  $\mu$ g of expression plasmid for Myc-tagged TRAF6 CT. The total amount of plasmid DNA was kept at 5.0  $\mu$ g by using an empty vector. Thirty-six hours after transfection, cell lysates were prepared, immunoprecipitated with anti-FLAG mAb (IP), and then immunoblotted with the indicated Abs (IB). The same lysates were simultaneously blotted with anti-Myc mAb to monitor TBK1 and TRAF6 CT expressions. *B*, The full length of TRAF6 also inhibits TRIF-induced IFN- $\beta$  promoter activation. 293 cells were transiently transfected with empty vector (mock) or expression vector for TRAF6, wild-type TRIF, TRIF 3A mutant, or the indicated combination, together with the IFN- $\beta$  promoter luciferase reporter. Thirty-six hours after transfection, luciferase activity was measured. The data are representative of three independent experiments.



**FIGURE 6.** TBK1 and TRAF6 do not share the same region of TRIF for the association. 293 cells ( $2 \times 10^6$ ) were transiently cotransfected with 2.5  $\mu\text{g}$  of expression plasmid for Myc-tagged TBK1 and FLAG-tagged TRIF $\Delta\text{C}$  or TRIF 3 $\Delta\Delta\text{C}$ . The total amount of plasmid DNA was kept at 5.0  $\mu\text{g}$  by using an empty vector. Thirty-six hours after transfection, whole cell lysates were prepared, immunoprecipitated with anti-FLAG mAb (IP), and then immunoblotted with the indicated Abs (IB). The same lysates were simultaneously immunoprecipitated and immunoblotted with anti-Myc mAb to monitor TBK1 expression.

cells in response to either LPS or poly(I:C) was normal in TIRAP-deficient mice (18, 19). Therefore, TRIF seems to be the most promising candidate responsible for the MyD88-independent pathway in TLR4 and TLR3 signaling.

In this study, we have identified TRIF-associated molecules by yeast two hybrid screening and a coimmunoprecipitation technique, and assessed the role of these molecules in the signaling cascades leading to the activation of NF- $\kappa\text{B}$  as well as the IFN- $\beta$  promoter. First, we showed that TRAF6 and TRIF interact physically and functionally through an association between the TRAF domain of TRAF6 and the N-terminal region of TRIF. Furthermore, we showed that disruption of the TRAF6-binding motifs of TRIF resulted in impairment of the association between TRIF and TRAF6 as well as TRIF-induced NF- $\kappa\text{B}$  activation (Fig. 2, C and D). Thus, it is likely that TRAF6 associates with TRIF directly, and this association induces NF- $\kappa\text{B}$  activation. In this regard, Jiang et al. (13) showed that TLR3 can regulate the activation of NF- $\kappa\text{B}$  through TRAF6 in IRAK-1-deficient cells, and neither MyD88 nor TIRAP was recruited to TLR3 in response to poly(I:C). In addition, we previously reported that TRIF associates with TLR3 (9). Taken together, these results suggested that one of the roles of TRIF is to link TLR3 and TRAF6 for NF- $\kappa\text{B}$  activation, independently of MyD88 and IRAK-1 (Fig. 7). However, we also pro-



**FIGURE 7.** Model of the signaling pathways through TRIF; detailed further in *Discussion*.

posed here that there might be a TRIF-dependent/TRAF6-independent pathway leading to NF- $\kappa\text{B}$  activation because both the TRIF 3A mutant and C-terminal half of TRIF, neither of which have TRAF6-binding motifs, possess the ability to activate NF- $\kappa\text{B}$  (Figs. 2C and 7). In fact, TLR4-mediated NF- $\kappa\text{B}$  activation was still observed in MyD88, TRAF6-doubly deficient cells, indicating that there is an as yet unidentified pathway, which results in the activation of NF- $\kappa\text{B}$  independently of MyD88 and TRAF6 (7).

More recently, Sharma et al. (14) and Fitzgerald et al. (15) showed that IKK $\beta$  and TBK1 act as IRF-3 kinases, and associate with not only IRF-3 but also TRIF. By coimmunoprecipitation assay, we have also found that TBK1, but not IKK $\beta$ , was associated with TRIF (Fig. 4B). We also showed that coexpression of TBK1 with TRIF induced phosphorylation of TRIF, as is the case of the endogenous TRIF modification in response to LPS, and this modification was dependent on the kinase activity of TBK1 (Fig. 1D and 4B). Furthermore, TBK1-associated TRIF is a phosphorylated form (Fig. 4C). Collectively, TRIF, TBK1, and IRF-3 form a complex, which was dependent on TBK1 kinase activity (Fig. 7). Although we could not detect TRIF-IKK $\beta$  interaction, TRIF was also phosphorylated when IKK $\beta$  was coexpressed, as is the case with TBK1 coexpression (Fig. 4B). Hence, it is possible that IKK $\beta$  associates with TRIF indirectly, and modulates TRIF through some endogenous signaling molecule(s). One such candidate is TANK/I-TRAF, which has been reported to interact with both IKK $\beta$  and TBK1 (20–22). However, we could not detect interaction between TRIF and TANK/I-TRAF in a coimmunoprecipitation assay (our unpublished data). These results suggest that TANK/I-TRAF is not involved in the association between TRIF and IKK $\beta$ , and/or another molecule is responsible for this interaction.

Our observations showing that TBK1 associates with the N-terminal portion of TRIF are consistent with our previous report in which the N-terminal, but not the C-terminal, truncated TRIF failed to activate the IFN- $\beta$  promoter, and with the fact that ectopic expression of TBK1 activates the IFN- $\beta$  promoter (Refs. 9, 14 and 15 and Fig. 4A). Although TRAF6 and TBK1 might not share the regions of TRIF to associate with it, it seems that TRAF6 physically blocks the association between TRIF and TBK1 because the increased expression of the TRAF domain of TRAF6 repressed the association of TRIF-TBK1. Moreover, not only the TRAF domain but also the full length of TRAF6 inhibited TRIF-induced IFN- $\beta$  promoter activation (Fig. 5B).

Recently, we have generated and analyzed the TRIF-deficient mice, and found that TRIF is essential for TLR3- and TLR4-mediated signaling pathways (23). In TLR4 signaling, we also demonstrated that TRIF might be involved in the “late phase” but not “early phase”, activation of NF- $\kappa\text{B}$  as well as the activation of IRF-3. Hence, TRAF6 might mediate both early and late phase activation of NF- $\kappa\text{B}$  in response to LPS, cooperating with MyD88 and TRIF, respectively.

In this paper, we demonstrated that TRAF6 and TBK1 are involved in TRIF-induced signaling cascades leading to the activation of NF- $\kappa\text{B}$  and IFN- $\beta$ , respectively. However, another pathway leading to the activation of NF- $\kappa\text{B}$ , which is triggered by the C-terminal domain of TRIF, remains to be elucidated. Additional experiments will be required for understanding the TRIF-induced activation of NF- $\kappa\text{B}$ .

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