Induction of type I interferons can be triggered by viral components through Toll-like receptors or intracellular viral receptors such as retinoic acid-inducible gene I. Here, we demonstrate that the TRAF (tumor necrosis factor receptor-associated factor) family member-associated NF-κB activator (TANK) plays an important role in interferon induction through both retinoic acid-inducible gene I- and Toll-like receptor-dependent pathways. TANK forms complexes with both upstream signal mediators, such as Cardif/MAVS/IPS-1/VISA, TRIF (Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β), and TRAF3 and downstream mediators TANK-binding kinase 1, inducible IκB kinase, and interferon regulatory factor 3. In addition, it synergizes with these signaling components in interferon induction. Specific knockdown of TANK results in reduced type I interferon production, increased viral titers, and enhanced cell sensitivity to viral infection. Thus, TANK may be a critical adaptor that regulates the assembly of the TANK-binding kinase 1-inducible IκB kinase complex with upstream signaling molecules in multiple antiviral pathways.

A striking consequence of viral infection as well as Toll-like receptor (TLR) stimulation is the induction of type I IFNs that include IFNβ and more than a dozen IFNα proteins. These cytokines bind to a common receptor, the type I IFN receptor (IFNAR), leading to induction of a large set of genes important in antiviral responses (1–3). Induction of IFN is triggered by microbial components termed pathogen-associated molecular patterns, such as viral RNA and DNA, which can be viewed as a molecular “signature” of the invading pathogens (4–7). A major class of cellular proteins capable of recognizing pathogens is the family of membrane receptors termed Toll-like receptors (4–6). In addition, many types of cells also have TLR-independent, intracellular detection systems to sense viral invasion and initiate innate immune responses (8–10). Recent studies have identified an RNA helicase-CARD-containing protein RIG-I and its homologue, Helicard/Mda5, as major intracellular receptors for viral double-stranded RNA.

Upon recognition of different subsets of pathogen-associated molecular patterns, individual TLRs trigger distinct innate immune responses via recruitment of different MyD88 adaptor family members, primarily MyD88 and Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF) (5, 11–14). These adaptors function as a platform to organize downstream molecules into signaling complexes, leading to activation of multiple signal cascades, particularly the NF-κB and IFN pathways, eventually resulting in specific cellular responses against different types of pathogens. Activation of NF-κB pathway requires the IκB kinase (IKK) signalosome, a signaling complex composed of two closely related kinase subunits (IκKα and IκKβ) and a regulatory subunit NF-κB essential modulator (NEMO, also called IκKγ) (15–18). IFN induction by both TLR and RIG-I pathways requires the TANK-binding kinase 1 (TBK1) or inducible IκB kinase (IκKι), two non-canonical members of the IKK family (19–23) that have been shown to function as the primary kinases capable of phosphorylating interferon regulatory factor 3 and 7 (IRF3/IRF7). TBK1- and IκKι-mediated phosphorylation and activation of IRF3 and IRF7 are critical steps for IFN production during TLR stimulation or viral infection (19–21, 24). Among 11 known TLR family members (1, 2, 6, 8, 13, 19, 25), TLR3 and TLR4-dependent IRF3/IRF7 phosphorylation and IFN induction require the adaptor protein TRIF, whereas TLR7/8 and TLR9-mediated activation of IFNs depends on MyD88 (2, 6, 13, 25, 26). However, the connection between TRIF or MyD88 and TBLK1-IκKι in TLR-mediated signal transduction pathways for type I interferon production is not yet fully understood. In RIG-I-mediated intracellular viral detection pathway, the helicase domain of RIG-I can recognize the viral double-stranded RNA. The N-terminal CARD regions can induce NF-κB and IRF3/IRF7 activation (8–10, 27–30). Recent reporters also demonstrated that Cardif/MAVS/IPS-1/VISA functions as a primary adaptor protein for RIG-I in IFN induction pathways (31–34), but it is not yet clear how the Cardif/MAVS/IPS-1/VISA induces IFN activation. Recently, we found that TRAF3 plays a critical role in TLR as well as viral-induced IFN activation (35,
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FIGURE 1. TANK promotes type I IFN activation and cellular anti-viral activities. A–C, 293T cells were transfected with increasing amounts of a plasmid encoding TANK and a luciferase reporter plasmid carrying IFNβ, IFN-stimulated response element, or NF-κB promoter as well as a pCMV-LacZ plasmid as a transfection control. 36 h after transfection the luciferase activities were measured and normalized based upon β-galactosidase activity. Data are expressed as the -fold induction calculated for each sample by dividing the luciferase activity by that observed in the sample containing only empty expression vectors. Data are represented as the means ± S.D. of duplicate samples and are representative of four independent experiments. D, 293T cells were transiently transfected with TANK expression plasmid. 24 h after transfection IFNβ levels in culture supernatants were determined by IFN ELISA. Results are reported as the means ± S.D. of duplicate samples from one representative experiment of three independent experiments. E, effect of anti-IFNα/β antibodies (Ab) on TANK-mediated STAT1 phosphorylation (pSTAT1). 293T cells were transfected with 500 ng of TANK expression vector; 36 h after transfection, the culture supernatant was collected as cell-free CM. Then the CM were treated with a mixture of neutralizing anti-IFNα and anti-IFNβ antibodies or nonspecific mouse IgG for 1 h and were added onto fresh 293 cells for 1 h. CM-induced STAT1 phosphorylation was analyzed by Western blot. Similar results were obtained in three independent experiments. F, 293T cells were transfected with FLAG-tagged TANK. 24 h after transfection cells were infected with VSV at a multiplicity of infection of 0.1 for 24 h. The viral titer was measured by plaque assay. Representative data from one of three experiments are shown.

36). Our studies also show that TAF3 can interact with MyD88, TRIF, and Cardif (35, 37).

Because both TBK1 and IKKi are able to interact with an adaptor protein TANK (38, 39), it is possible that TANK may participate in IFN induction pathways by organizing TBK1-IKKi signaling complexes. TANK was originally identified as a protein associated with TRAF3 (40, 41), but its function has remained elusive. No studies have been reported on the function of either TANK itself or the TANK-TBK1-IKKi complex in IFN induction. Recent findings on the role of TBK1-IKKi as well as TRAF3 in IFN induction shed new light on the function of TANK. Results from this study indicate that TANK might be an important modulator of type I IFN induction in both TLR-dependent and intracellular detection mechanisms during viral infections.

EXPERIMENTAL PROCEDURES

Reagents—Poly(IC) was purchased from Amersham Biosciences. LPS was purchased from Sigma Aldrich. Antibody reagents included anti-Myc and anti-FLAG (Sigma-Aldrich), anti-TANK, anti-TBK, anti-IKKi, anti-IKβ, anti-IRF3, and anti-TRAFl (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-TANK (Biovision, Mountain View, CA), anti-Cardif (AXXORA, San Diego, CA), anti-IRF3 (Zymed Laboratories Inc., San Francisco, CA), and anti-pSTAT1, anti-phospho-extracellular signal-regulated kinase, and pp38 (Cell Signaling, Beverly, MA), anti-TBK (Upstate Biotechnology, Inc., Lake Placid, NY). FLAG-TANK, HA-TRAFl, HA-TBK1, and HA-IKKi constructs were described previously. Full-length TRIF and Mda5 was cloned into Myc-pCMV eukaryotic expression vector. RIG-I construct was a gift from T. Fujita.

Murine macrophage cell line RAW264.7, mouse embryonic fibroblasts (MEFs) and 293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Murine bone marrow-derived macrophages (BMMs) were generated by culturing the bone marrow cells for 7 days in media containing macrophage colony-stimulating factor.

Reporter Gene Assays—Luciferase assays were performed as previously described (42, 43). Briefly, 293T cells seeded on 12-well plates were transiently transfected with a luciferase reporter plasmid, and various expression plasmids were indicated. In each experiment total amounts of DNA in each sample were kept constant by supplementation with the appropriate empty parental expression vector(s). For normalization of transfection efficiency, each transfection included 50 ng of a pCMV-LacZ control plasmid, which constitutively expresses β-galactosidase. 36 h after transfection cells were harvested, and firefly luciferase activities were analyzed using a luciferase assay kit (Promega Corp.) according to the manufacturer’s protocol. -Fold induction was calculated for each sample by dividing the luciferase activity, normalized to β-galactosidase activity, by that observed in the sample containing only empty parental expression vectors. To measure luciferase activity in RAW cells, RAW cells seeded on 6-well plates were transfected with plasmids indicated, and 24 h after transfection cells were stimulated with LPS or poly(IC) or infected with virus for 24 h, and luciferase activities were measured.

Immunoblotting and Immunoprecipitation—293T cell transfection was performed as described (42, 43). For Western analyses, protein samples in modified radioimmune precipitation...
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**Assay Buffer Separation**

Assay buffer were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using standard methods. For immunoprecipitation, total cell lysates were pre-cleared with Sepharose protein G beads for 1 h at 4°C. The cell lysates were immunoprecipitated with 2 μg of specific antibody or control IgG for 16 h. The immunoprecipitates were washed 3 times with the lysis buffer and analyzed by Western blot.

**RNA Interference**

In siRNA experiments, double-stranded RNA duplexes composed of 21-nucleotide oligonucleotides were synthesized by Invitrogen. The siRNA oligonucleotides used for targeting murine TANK was GAC UUU CUG GGA CCU UAA ATT. The siRNA oligonucleotide targeting human TANK was UCA CUU CAA CAG ACU AUU ATT. siRNA duplexes were transfected into cells in 6-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. GFP siRNA was used as a control. 48 h after transfection cell lines were used for further experiments. To examine the effect of silencing TANK on IFN reporter gene activity in RAW cells, the oligonucleotides targeting murine TANK sequences were cloned into expression plasmid pB5-hH1-3 as described (44) and transfected into RAW cells.

**Vesicular Stomatitis Virus (VSV) Infection and Plaque Assay**

For TANK-induced anti-viral response, 293T cells in 12-well plate were transfected with empty vector or pcMV-FLAG-TANK plasmid. 24 h after transfection cell lines were infected with VSV at a multiplicity of infection of 0.1 for 24 h. To examine the effect of silencing TANK on IFN induction, MEFs were transfected with a VSV reporter oligo duplexes for TANK or GFP using Lipofectamine 2000 (Invitrogen), and 48 h after siRNA treatment MEFs were infected with VSV at a multiplicity of 0.1. Cell viability was determined by the trypsin blue exclusion method. Virus yield was measured in culture supernatants collected from VSV-infected 293T cells or MEFs by standard plaque assay. Briefly, Vero cells were infected with serial dilutions of recovered viruses for 1 h and were overlaid with Dulbecco’s modified Eagle’s medium containing 0.5% low melting agarose. After 24 h of incubation, plates were stained with crystal violet, and plaques were counted.

**IFN Production**

To measure IFN production, 293T cells transfected with plasmids or infected with viruses were cultured for 24 h. Culture supernatants were collected, and IFN levels were determined by IFN ELISA kits according to the manufacturer’s instructions.

**Neutralizing Type I IFN Activities**

To block IFN activities in culture supernatants, an anti-human IFNα antibody and an anti-human IFNβ antibody (PBL Biomedical Laboratories) were used for targeting murine TANK.
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**RESULTS**

**TANK Is Able to Induce IFN Activation**—As an initial step to determine whether TANK is involved in the type I IFN activation pathway, we analyzed the effect of TANK on activation of IFN promoter. As shown in Fig. 1, A–C, overexpression of TANK alone was able to induce considerable IFN transcription in a dosage-dependent manner as measured by a reporter plasmid carrying either an IFNβ or IFN-stimulated response element promoter, which is regulated by IRFs. In accordance with early publications, TANK itself could slightly induce NF-κB transcription. We also confirmed the role of TANK in the induction of type I IFN at protein level. Production of endogenous IFNβ could be detected in the culture supernatants of 293T cells transfected with TANK in a dosage-dependent manner, although we could not detect IFN with lower concentrations of TANK, probably because the amount of IFN produced might be below the detection limit of the ELISA system. As an alternative way to examine the type I IFNs in the culture supernatants, we investigated whether type I IFNs induced by TANK could lead to the activation of STAT1. We conducted experiments using a mixture of anti-IFNα/β antibodies to neutralize IFN activities in the culture supernatants. As shown in Fig. 1E, the addition of conditional media (CM) collected from TANK-transfected cells led to STAT1 phosphorylation, as opposed to CM from cells transfected with empty vector. Treatment using anti-IFNα/β antibodies but not nonspecific control IgG clearly reduced STAT1 activation, indicating that type I IFNs in the culture supernatants were responsible for this effect.

Because type I IFNs provide immediate innate antiviral defense, we then analyzed the effect of TANK-induced IFN on VSV infection. VSV is a cytopathic virus and is very sensitive to IFNs and has been widely used in IFN antiviral assays (19, 33, 34, 45). 293T cells were transfected with an empty vector or a plasmid encoding TANK. Subsequently, cells were infected with VSV for 24 h, and viral titer was measured by plaque assay. As shown in Fig. 1F, overexpression of TANK reduced virus growth as supernatant from TANK-expressing cells had much lower viral titer. These results indicate that TANK has the ability to furnish antiviral responses through induction of type I IFN.

**Involvement of TANK in Multiple Viral Detection Pathways**—To more directly investigate the involvement of TANK in virally induced type I IFN production, we examined the effect of silencing of TANK on IFNβ induction by viral infection. As fibroblast cells, 293T cells lack most of the TLRs; therefore, IFN response is likely mediated through intracellular detection pathways. Knockdown of endogenous TANK protein in 293T cells decreased IFNβ transcription induced by Sendai virus infection or transfection of poly(IC), which has been shown to mimic viral double-stranded RNA (Fig. 2A). Consequently, 293T cells treated with TANK siRNA, but not GFP siRNA, produced much less IFN protein in response to poly(IC) transfection and Sendai virus infection (Fig. 2B). The efficiency of siRNA to reduce TANK protein levels in human and murine cells is shown in Fig. 2C. We further elucidated TANK-mediated STAT1 phosphorylation triggered by viral infection. Although viral infection induced strong STAT1 phosphorylation in cells with control siRNA, silencing TANK expression led to impaired STAT1 phosphorylation during viral infection (Fig. 2D). To further confirm the role of type I IFN in TANK-dependent phosphorylation of STAT1, we utilized a neutralizing antibody against IFNAR to block binding of type I IFNs with their receptor. The addition of the neutralizing anti-human IFNAR antibody but not isotype control significantly inhibited...
viral induced STAT1 phosphorylation (Fig. 2E). These data together demonstrate that type I IFNs induced by TANK was responsible for TANK-mediated STAT1 phosphorylation.

We also monitored viral-induced STAT1 phosphorylation in murine BMMs, which may use TLR-dependent and -independent mechanisms to detect viral infection. As shown in Fig. 2F, knocking down TANK protein severely reduced virally induced STAT1 phosphorylation. Next, we investigated the involvement of TANK in intracellular anti-viral response in MEFs. MEFs treated with siRNA duplexes specific for TANK were infected with VSV. TBK1-deficient MEFs were also included as a positive control. As shown in Fig. 3, almost all of TBK1-deficient cells died post-VSV infection because of defects in IFN induction. However, IFN pretreatment could reduce such VSV-induced cell death. As for TBK1-deficient cells, but with a lesser extent, MEFs treated with siRNA duplexes specific for TANK also exhibited accelerated cell death upon VSV infection (Fig. 3, A and C). Accordingly, MEFs with knockdown of TANK had much higher viral titer (Fig. 3B). These data point out that TANK may play an important role in innate immunity against viral infection.

Association of TANK with the Adaptor Protein Cardif—Induction of type I interferons in fibroblasts is triggered by intracellular viral receptors such as RIG-I. Recent studies demonstrate that Cardif/MAVS/IPS-1/VISA is a critical adaptor protein for the RIG-I-mediated NF-κB and IFN activation. To further elucidate the mechanism of TANK-mediated IFN induction by viral infection, we examined the involvement of TANK in RIG-I pathways. We observed that knockdown of TANK protein inhibited activation of type I IFN promoters by RIG-I (Fig. 4A). We also found that TANK could synergize with Cardif to induce IFN promoter transcription (Fig. 4B). Conversely, knockdown of TANK reduced IFNβ production induced by Cardif (Fig. 4C). Furthermore, TANK and Mda5/Helicard, a homologue of RIG-I, together had a strong synergy with TBK1 in IFN induction (data not shown). These data suggest that the TANK may participate in cytoplasmic detection pathways.

The involvement of TANK in the RIG-I pathway promoted us to examine the interaction between TANK and signaling molecules in this pathway. Although we were unable to detect an interaction between TANK and RIG-I in our overexpression system, we did find interaction of TANK with the adaptor protein Cardif/MAVS/IPS-1/VISA. When cell lysates from 293T cells transfected with FLAG-TANK and Myc-Cardif were co-expressed in 293T in 12-well for 36 h, and luciferase reporter activities were measured and normalized based upon β-galactosidase activity. Data are expressed as the -fold induction calculated for each sample by dividing the luciferase activity by that observed in the sample containing only empty expression vectors. Data are represented as the means ± S.D. of duplicate samples. B, TANK-enhanced Cardif-mediated IFN activation. 50 ng of TANK and 10 ng of Cardif were co-expressed in 293T in 12-well for 36 h, and induction of IFNβ and IFNα transcription activities were measured by luciferase assay as described under “Experimental Procedures.” C, 293T cells treated with TANK siRNA or GFP siRNA oligo duplexes were transiently transfected with 250 ng of Cardif. After 24 h culture supernatant was collected and analyzed by ELISA for production of IFNβ. Data represent as the means ± S.D. of duplicate samples. D, 293T cells were transfected with 2.5 μg each of FLAG-TANK and Myc-Cardif as indicated. 36 h after transfection cell lysates were immunoprecipitated with anti-FLAG or anti-Myc antibody. The immunoprecipitates (IP) were probed by Western blot (WB) with anti-Myc or anti-FLAG antibodies. The cell lysates were also analyzed by Western blot using anti-FLAG and anti-Myc antibodies. Data are representative of four independent experiments (A and B) or three independent experiments (C and D).

FIGURE 4. TANK is involved in RIG-I-dependent IFN induction pathways. A, TANK siRNA diminished RIG-I-induced type I IFN activation. 293T cells treated with siRNA oligo duplexes targeting TANK or GFP were transiently transfected with 25 ng of RIG-I. 36 h after transfection, and the luciferase activities were measured and normalized based upon β-galactosidase activity. Data are expressed as the -fold induction calculated for each sample by dividing the luciferase activity by that observed in the sample containing only empty expression vectors. Data are represented as the means ± S.D. of duplicate samples. B, TANK-enhanced Cardif-mediated IFN activation. 50 ng of TANK and 10 ng of Cardif were co-expressed in 293T in 12-well for 36 h, and induction of IFNβ and IFNα transcription activities were measured by luciferase assay as described under “Experimental Procedures.” C, 293T cells treated with TANK siRNA or GFP siRNA oligo duplexes were transiently transfected with 250 ng of Cardif. After 24 h culture supernatant was collected and analyzed by ELISA for production of IFNβ. Data represent as the means ± S.D. of duplicate samples. D, 293T cells were transfected with 2.5 μg each of FLAG-TANK and Myc-Cardif as indicated. 36 h after transfection cell lysates were immunoprecipitated with anti-FLAG or anti-Myc antibody. The immunoprecipitates (IP) were probed by Western blot (WB) with anti-Myc or anti-FLAG antibodies. The cell lysates were also analyzed by Western blot using anti-FLAG and anti-Myc antibodies. Data are representative of four independent experiments (A and B) or three independent experiments (C and D).
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**FIGURE 5. Knockdown of endogenous TANK abrogates TLR-mediated IFN induction.** A, RAW cells were transfected with an expression plasmid encoding TANK and an IFNβ luciferase plasmid. 24 h after transfection cells were treated with LPS (100 ng/ml) or poly(IC) (1 μg/ml) for 24 h. The luciferase activities were measured and normalized to β-galactosidase activity as described under “Experimental Procedures.” Data are shown as -fold induction relative to the unstimulated vector control in each experiment and mean values ± S.D. of duplicate samples. B, RAW cells were transfected with plasmids expressing RNA interference specific for murine TANK or GFP and a luciferase reporter plasmid, and 48 h after transfection cells were stimulated with LPS (100 ng/ml), CpG (100 nM), or poly(IC) (1 μg/ml), or R848 (1 ng/ml) to stimulate TLR4, -9, -3, and -7/8, respectively, and induction of IFNβ promoter activity was measured by luciferase assay as described under “Experimental Procedures.” Data are shown as -fold induction relative to the unstimulated vector control in each experiment and mean values ± S.D. of duplicate samples. C, RAW cells treated with TANK siRNA or GFP siRNA were stimulated with 100 ng/ml LPS for indicated times and analyzed for STAT1 phosphorylation by Western blot. As the control, cell lysates were also blotted for STAT1 protein, extracellular signal-regulated kinase (ERK) phosphorylation, and ERK protein. D, knockdown of TANK in primary macrophages. siRNA oligos targeting TANK or GFP were transfected into BMMs to silence the expression of endogenous TANK protein. 48 h after transfection cells were stimulated with LPS for indicated time. Cell lysates were immunoblotted with antibodies against phospho-STAT1, phospho-p38, p38 protein, and STAT1 protein. E, similar to D except that BMMs were stimulated with poly(IC). Cell lysates were blotted for phospho-STAT1 and STAT1 protein. All data are representative of three-four independent experiments.

were treated with TANK siRNA or control GFP siRNA, then stimulated with LPS, CpG, poly(IC), or R848 to activate TLR4, -9, -3, or -7 respectively. Knocking down TANK protein led to a significant reduction in IFNβ promoter transcription induced by various TLRs (Fig. 5B). Consequently, silencing TANK protein expression also resulted in impaired STAT1 phosphorylation in RAW cells stimulated with LPS (Fig. 5C). Compared with the IFN pathway, TLR-induced extracellular signal-regulated kinase activation was generally not affected.

We further extended these studies to primary macrophages. BMMs were transfected with siRNA to knock down TANK protein expression, and STAT1 phosphorylation was determined after stimulation with LPS or poly(IC). Knockdown of TANK significantly impaired TLR3- and TLR4-induced STAT1 phosphorylation while showing little or no effects on either p38 or c-Jun NH2-terminal kinase activation (Fig. 5, D and E, and data not shown). These results imply that TANK might play an important role in the type I IFN induction in macrophages in response to multiple TLR ligands.

**TANK Cooperates with TRAF3 and TRIF in IFN Induction**—Induction of type I interferons triggered by LPS or poly(IC) is mediated by the adaptor protein TRIF. We, therefore, evaluated the contribution of TANK to the TRIF-dependent IFN pathway. Fig. 6A shows that TANK could enhance TRIF-mediated IFN activation. Conversely, knockdown of TANK diminished TRIF-induced IFNβ and IFN-stimulated response element promoter activation (Fig. 6B). These results indicate that TANK and TRIF could synergistically stimulate IFN expression in TLR pathways.

Because TANK was originally identified via yeast two-hybrid screening as a TRAF3-interacting protein (42, 46) and TRAF3-deficient cells have defective IFN response to TLR stimulation and viral infection (35, 36), we hypothesized that TANK in association with TRAF3 may provide a molecular link between TRIF and downstream signaling molecules. Even though either TANK or TRAF3 alone is a weak inducer for IFNβ production, TANK and TRAF3 exhibited a great synergy in enhancing TRIF-mediated IFNβ induction (Fig. 6C). The requirement for TANK and TRAF3 in IFN activation and their cooperation with TRIF suggested that these signaling molecules may interact with each other. Therefore, we performed immunoprecipitation studies to evaluate this possibility. Myc-tagged TRIF was co-expressed with TRAF3 and TANK and was immunoprecipitated using anti-Myc antibody. Then immunoprecipitates were blotted with anti-TANK or anti-TRAF3-specific antibodies. TANK and TRAF3 could be detected in TRIF immunoprecipitates. Interestingly, the amount of TANK associated with TRAF3 evidently increased when TRAF3 was co-expressed (Fig. 6D). These results suggest these signaling proteins may form a supramolecular complex.

**TANK Interacts with TBK1 and IKKi in IFN Induction Pathways**—Both TLR and RIG-I pathways utilize TBK1 and/or IKKi kinases for IFN induction. TBK1 was initially identified through its interaction with TANK in yeast two-hybrid screening. Subsequent studies also revealed the interaction between TANK and IKKi (38, 39, 47). The ability of TANK to interact with TBK1-IKKi and the involvement of TANK in TRIF- and Cardif-mediated IFN activation imply that TANK may interact...
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and modulate TBK1-IKKi activities. To test this, we examined whether TANK cooperates with TBK1 and IKKi in the type I IFN induction pathway. 293T cells were transiently transfected with TANK and TBK1 or IKKi expression plasmids, and induction of IFNβ was measured by reporter gene assay. TANK was found to be able to increase TBK1- and IKKi-induced IFNβ promoter activities (Fig. 7, A and B). In agreement with previous overexpression studies, we found TANK could interact with TBK1 and IKKi in the 293T overexpression system (Fig. 7C and data not shown). Our data further demonstrate that TANK could form a signaling complex with TBK1 and IRF3 (Fig. 7C). When FLAG-tagged TANK was immunoprecipitated with anti-FLAG antibody, co-expressed TBK1 and IRF3 were detected by Western blot analysis. Of note, immunoblotting displayed that endogenous TBK1 protein could be immunoprecipitated with TANK in 293T cells (Fig. 7C, lane 2). Our results imply that TANK could potentially function as an adaptor molecule to organize and collaborate with TBK1 and IKKi in IFN activation.

TANK Is Recruited to Upstream Signaling Complexes in Response to Viral Infection or TLR Stimulation—The results described above promoted us to examine interaction of TANK with these signaling molecules under physiological conditions. First, we analyzed endogenous TANK signaling complexes in viral infected cells. MEFs were infected with Sendai virus, and TANK signaling complexes were immunoprecipitated with an anti-TANK antibody. A number of signaling molecules including Cardif, TRAF3, and TBK1 were found to associate with TANK. Interestingly, increased association between TANK and Cardif was observed in viral-infected MEFs (Fig. 7D). Together, our results demonstrate the formation of Cardif/TRAF3/TANK/TBK1 signaling complex during viral infection.

We then analyzed endogenous TANK signaling complexes in macrophages. The signaling complex was immunoprecipitated from untreated or poly(I:C)-stimulated macrophages using anti-TRAF3 antibody. TANK was detected in TRAF3 immunoprecipitates from both stimulated and non-stimulated RAW cells (Fig. 7E). No association was observed using control antibodies. The relative amount of TBK1 in TRAF3 immunoprecipitates increased upon TLR stimulation. We further investigated the recruitment of signaling molecules to TRIF. A number of signaling molecules including TANK, TBK1, and IRF3 were found to associate with the adaptor TRIF, and such association was increased upon poly(I:C) stimulation in macrophages (Fig. 7F). These results suggest that TLR stimulation leads to further recruitment of these proteins to form a TRIF signaling complex essential for IFN activation. Collectively, our data imply that TANK could function as an adaptor molecule to link and recruit downstream TBK1-IKKi to the upstream signaling complex in multiple IFN induction pathways.

DISCUSSION

Induction of type I IFNs is triggered by microbial components through TLR or a variety of TLR-independent intracellular pattern recognition receptors such as RIG-I. A critical step essential for both recognition systems is TBK1- and IKKi-induced phosphorylation and activation of IRF3 and IRF7. However, the molecular mechanisms responsible for activation of TBK1-IKKi kinase activities are not fully understood. In this study we demonstrate that TANK might provide the link between upstream molecules, such as TRIF or Cardif, and the downstream signaling complex TBK1-IKKi-IRF3. The specific role played by TANK in TBK1-IKKi activation and IFN induction may resemble the critical role of NEMO in regulating
TANK provides molecular link between TBK1-IKKi and upstream signaling complexes in the IFN pathway. A and B, 293T cells were transfected with TBK1 (50 ng) or IKKi (50 ng) and increasing amounts of TANK. 36 h after transfection the luciferase activities were measured and normalized based upon β-galactosidase activity. Data are expressed as the fold induction calculated for each sample by dividing the luciferase activity by that observed in the sample containing only empty expression vectors. Data represent the means ± S.D. of duplicate samples and are representative of four independent experiments. C, 293T cells were transfected with FLAG-TANK, HA-TBK1, and HA-IRF3 as indicated. 36 h after transfection cell lysates were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were probed by Western blot (WB) with anti-TBK1, anti-HA for IRF3, and anti-TANK antibodies. D, MEFs were infected with Sendai virus for 2 and 6 h, and cell lysates were immunoprecipitated with control goat IgG or anti-TANK antibody. The immunoprecipitates were analyzed by Western blot with antibodies specific for Cardif, TANK, TRAF3, and TBK1. E, endogenous interactions of TRAF3, TANK, and TBK1 in macrophages. RAW cells were stimulated with or without 1 μg/ml of poly(I:C) for 4 h. The cell lysates were immunoprecipitated with anti-TRAF3 antibody or control rabbit IgG. The immunoprecipitates were analyzed by Western blot with antibodies specific for TANK, TBK1, and TRAF3. HC, heavy chain. F, endogenous association of TRAF3, TANK, TBK1, and IRF3 with TRIF. RAW cells were stimulated with poly(I:C). The cell lysates were immunoprecipitated with anti-TRIF antibody. The immunoprecipitates were analyzed by Western blot with antibodies specific for TRAF3, TANK, TBK1, IRF3, and TRIF. All data in C–F are representative of three independent experiments.

Numerous studies indicated that, depending upon the nature of viruses or the cell types infected by viruses, host cells can recognize viral infection and produce type I interferons either by TLRs in innate immune cells such as macrophages and dendritic cells or various intracellular pattern recognition receptors in most cell types. Recent studies indicate that CARD-containing proteins RIG-I and Helicard/Mda5 play a key role in detection of viral double-stranded RNA. The binding of viral RNA to the helicase domain potentially induces a conformation change in RIG-I protein that leads to recruitment of downstream signaling molecules via its CARD domains, thereby triggering the activation of NF-κB and IRF3. The importance of RIG-I has been demonstrated in genetic studies as RIG-I-deficient fibroblasts fail to produce IFNβ in response to viral infection. Recently, several groups have identified a novel CARD-containing adaptor protein, variously termed IPS-1, MAVS, VISA, or Cardif, as a downstream adaptor molecule of RIG-I (31–34). Moreover, Cardif has been shown to be targeted by hepatitis C virus to evade innate immune systems. Despite these exciting findings, there are significant inconsistencies regarding Cardif-interacting proteins. Therefore, the mechanisms by which Cardif activates NF-κB and IFN pathways remain largely unknown. In the current studies we demonstrated the interaction between TANK and Cardif. Our data also show the association of endogenous TANK and other signaling molecules such as TBK1 with Cardif during Sendai virus infection. Although it remains to be determined if TANK directly binds to Cardif or through adaptor proteins such as TRAF family members, it seems that TANK may link RIG-I/Cardif to TBK1-IKKi/IRFs complexes. Thus, TANK may modulate or orchestrate signaling events essential for IFN activation in response to viral infection.

Interestingly, our data also indicate that TANK is involved in IFNβ induction by TLRs. TRIF has been demonstrated to be the major adaptor molecule for TLR3 or TLR4-mediated type I IFN induction (5, 12–14). Our work suggests that the TANK-TBK1-IKKi complex may be recruited to TRIF via another adaptor molecule, TRAF3. We found TANK could associate with TRIF and TRAF3 and collaborate with these molecules in inducing IFNβ promoter activation. This finding is contradic-
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In summary, the results presented in this study implicate TANK as a key adaptor protein that converges signals generated from both membrane receptors, such as TLRs, and cytoplasmic sensors, such as RIG-I, into the TBK1-IKKi signaling complex, eventually leading to phosphorylation of IRF3/7 and production of type I IFNs. These data suggest that TANK may be an important component in innate immune responses. This also raises the possibility that TLRs and intracellular detection receptors may modulate each other’s signaling pathways through the TANK/TBK1/I KK complex in response to viral infection.

Acknowledgments—We thank Dr. Takashi Fujita for the RIG-I plasmid. We thank members of our laboratory for discussion and for reading this manuscript.

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