Recognition of viral RNA by Toll-like receptor 3 (TLR3) triggers activation of the transcription factors NF-κB and IRF3 and induction of type I interferons. TRIF is a Toll-interleukin 1 receptor (TIR) domain-containing adapter protein critically involved in TLR3-mediated signaling. It has been shown that TRIF interacts with TLR3 through their respective TIR domains. In this study, we identified a splice variant of TRIF lacking the TIR domain, which is designated as TRIS. Overexpression of TRIS activates NF-κB, interferon-stimulated response element (ISRE), and the interferon-β promoter, whereas knockdown of TRIS inhibited TLR3-mediated signaling, suggesting that TRIS is involved in TLR3-mediated signaling. Furthermore, we identified an N-terminal TBK1-binding motif of TRIS or TRIF that was important for its interaction with TBK1 and ability to activate ISRE. Activation of ISRE by TRIS also needs its dimerization or oligomerization mediated by its C-terminal RIP homotypic interaction motif. Finally, we demonstrated that TRIS was associated with TRIF upon TLR3 activation by poly(I-C). These findings reveal an unexpected mechanism of TLR3-mediated signaling.

Recognition of pathogen-associated molecular patterns by host pattern recognition receptors represents a critical step in innate immune response. In mammals, pathogen-associated molecular patterns are recognized by three classes of pattern recognition receptors, the membrane-bound Toll-like receptors (TLRs), cytosolic RIG-I-like receptors, and the Nod proteins. Detection of the pathogen-associated molecular patterns by pattern recognition receptors initiates a series of signaling events, leading to induction of various chemokines and cytokines and subsequent inflammatory and innate immune responses against the pathogens (1–3).

TLRs are evolutionarily conserved proteins that are critically involved in host defense from plants to humans (4, 5). The TLRs are characterized by a conserved cytoplasmic domain, the TIR domain, that is defined by a motif of ~160 amino acids including five β-sheets surrounded by α-helices and connected together by flexible loops (6). TLRs act as hetero- or homodimers that are thought to be pre-assembled in a low affinity complex under physiological conditions. Upon ligand binding, TLRs undergo conformational changes that bring the two TIR domains in the cytoplasmic regions into closer proximity, creating a new platform on which to build a signaling complex that triggers the activation of several transcription factors such as NF-κB, AP1, and IRF3 (7–9). These transcription factors cooperatively induce expression of downstream proteins involved in inflammation and innate immunity.

Among the TLRs, TLR3 has been shown to recognize viral double strand RNA. TLR3 signals through a TIR domain containing an adapter protein called TRIF/TICAM1 (10–12). TRIF-deficient mice are defective in TLR3-mediated activation of NF-κB and IRF3 and expression of type I IFNs and various proinflammatory cytokines, suggesting that TRIF is required for TLR3-mediated signaling (12, 13). TRIF contains an N-terminal domain, a middle TIR domain, and a C-terminal domain. It has been shown that the N-terminal domain of TRIF is associated with TBK1, probably through NAP1 and/or TRAF3, and is required for TRIF-mediated IRF3 activation (14–17). TRIF mediates NF-κB activation through two distinct pathways. TRIF contains a consensus TRAF6-binding motif in the N-terminal region, and mutation of this motif impairs TRIF-mediated NF-κB but not IRF3 activation (16, 18). However, the role of TRAF6 in TRIF signaling is still controversial as in TRAF6-deficient macrophages, in contrast to TRAF6-deficient mouse embryonic fibroblasts (MEFs), TRIF signaling was not affected (17–19). TRIF is also capable of activating NF-κB through its C-terminal RIP homotypic interaction motif (RHIM), which is responsible for recruiting RIP to TRIF (20). It has been demonstrated that poly(I-C)-induced NF-κB activation is completely blocked in RIP-deficient MEFs (21). In addition to NF-κB and IRF3 activation, TRIF is capable of inducing apoptosis. TRIF-induced apoptosis is mediated through RIP1, FADD, and caspase-8 (22, 23).

In this study, we identified a TIR-less splice variant of TRIF designated as TRIS. Rather than acting as an inhibitor of TLR3-mediated signaling, TRIS is surprisingly required for TLR3-mediated signaling. TRIS forms a heterocomplex with TRIF through their C-terminal RHIM motifs. Overexpression of TRIS activates NF-κB and IRF3, although specific knockdown of TRIS inhibits TLR3-mediated NF-κB and IRF3 activation. Our findings reveal an unexpected mechanism of TLR3-mediated signaling.
Signaling by a TIR-less Variant of TRIF

Identification of a TIR-less splice variant of TRIF. A, recognition of a 30-kDa cellular protein by anti-TRIF-N. Lysates from the indicated cell lines are shown. B, knockdown of TRIF but not the 30-kDa protein by TRIF-RNAi plasmids. 293 cells were transfected with the indicated RNAi plasmids for 36 h before immunoblot (IB) analysis with anti-TRIF-N was performed. Con, control. C, isolation of a splice variant of TRIF by PCR. Templates from a mixed cDNA library were amplified with TRIF-specific primer pairs as indicated. UTR, untranslated region. D, schematic representation of the position of TRIS-specific RNAi target sequences. E, knockdown of overexpressed (left panel) or endogenous (right panel) TRIS by TRIS-specific RNAi plasmids. Left panel, 293 cells were transfected with equal amounts of FLAG-TRIS, FLAG-TRIS, and FLAG-MPP5 (used as an internal control), together with a control GFP-RNAi plasmid or TRIS-RNAi plasmids. Thirty-six hours after transfection, cell lysates were analyzed by immunoblot with anti-FLAG. Right panel, 293 cells were transfected with a control GFP-RNAi or TRIS-RNAi plasmids for 36 h before immunoblot analysis with anti-TRIF-N was performed.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal antibodies against the FLAG and HA epitopes (Sigma), rabbit polyclonal anti-TRIF-N (catalog no. ALX-210-908, Alexis Biochemical), anti-TRIF (219) (catalog no. 4596, Cell Signaling Technology), 293, HeLa, and Jurkat cells (ATCC) were purchased from the indicated manufacturers. 293-TLR3 cells were provided by Drs. Katherine Fitzgerald and Tom Maniatis.

Constructs—The NF-κB, ISRE, and IFN-β promoter luciferase reporter plasmids and mammalian expression plasmids for TRIF and TRIF deletion mutants TBK1, TBK1-CA, IRF3-DN, and RIP were described previously (23, 24). Mammalian expression plasmids for TRIS, TRIS deletion and point mutants, TRIF and RIP were described previously (23, 24). Mammalian expression plasmids for TRIS, TRIS deletion and point mutants, TRIF and RIP were described previously (23, 24).

RNAi Experiments—Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper. Retro RNAi plasmid (Oligoengine Inc.). The target sequences for TRIF cDNA are as follows: 1) GACCAGACGCCACTCCAA; 2) GAAGATACCACCTCTCA; 3) TACCATGCCTTCCGCCAG. pSuper-Retro-GFP-RNAi plasmid (Oligoengine Inc.), which targets green fluorescent protein cDNA sequences, was used as a control for all RNAi-related experiments in this study.

Cell Transfection and Reporter Gene Assays—293 cells (2 × 10⁵) were seeded in 12-well dishes and transfected the following day by the calcium phosphate precipitation method. In the same experiment, each transfection was performed in triplicate, and where necessary, empty control plasmid was added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, 50 ng of pTK-β-galactosidase reporter plasmid was added to each transfection. Approximately 16 h after transfection, luciferase reporter assays were performed using a Dual-Luciferase(TM) assay kit (Promega) following the manufacturer’s protocol. Luciferase activities were normalized relative to β-galactosidase activities.

Coimmunoprecipitation and Immunoblot Analysis—For transient transfection and coimmunoprecipitation experiments, 293 cells (2 × 10⁶) were transfected for 24 h. Transfected cells were lysed in 1 ml of lysis buffer (15 mM Tris, 120 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5). For each immunoprecipitation, a 0.4-ml aliquot of lysate was incubated with 0.5 μg of the indicated monoclonal antibody or control mouse IgG and 20 μl of 1:1 slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) for 2 h. The Sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were fractionated by SDS-PAGE, and subsequent Western blot analysis was performed. All immunoprecipitation experiments were repeated at least twice, and similar data were obtained.

Gel Filtration Chromatography—293-TLR3 cells (2 × 10⁶) were treated with poly(I-C) or left untreated for 30 min. The cells were lysed in 1.5 ml of lysis buffer. The lysate was centrifuged for 1 h at 15,000 rpm. The supernatant was recovered and loaded on a Superdex 200 gel filtration chromatography column pre-equilibrated with lysis buffer. The samples were eluted from the column in lysis buffer at a flow rate of 0.5 ml/min and collected in fractions of 1 ml. The fractions were precipitated with 10% trichloroacetic acid and analyzed by Western blots with antibodies against the N terminus of TRIF/TRIS.

RESULTS

Identification of a TIR-less Splice Variant of TRIF—During our studies of the TRIF protein, we routinely found that a commercially available rabbit antibody raised against the N terminus of human TRIF (anti-TRIF-N) could detect an extra protein band of ~30 kDa in all examined cells, including 293, 293-TLR3 (a 293 cell line stably expressing TLR3), HeLa, and Jurkat T cells (Fig. 1A). The 30-kDa band was not cleaved or degraded TRIF,
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Because transfection of two independent TRIF RNAi plasmids down-regulated full-length TRIF but not the 30-kDa protein (Fig. 1B). The detection of the 30-kDa band could be due to nonspecific recognition by the antibody or, alternatively, represented a splice variant of TRIF. To distinguish these two possibilities, we isolated potential TRIF variants from a mixed cDNA library under PCR conditions favoring amplifying short cDNAs (Fig. 1C). Sequence analysis of the PCR products revealed the existence of a splice variant of TRIF lacking amino acids 218–657 of the full-length TRIF (Fig. 1D). Because this splice variant of TRIF lacks the TIR domain, which is located between amino acids 394 and 532 of full-length TRIF, we designated this variant as TRIS. The deduced molecular mass of amino acids 218–657 of the full-length TRIF (Fig. 1D). These data suggest that the 30-kDa band represents the TIR-less splice variant TRIS.

TRIS Is Required for TLR3-mediated Signaling—Various studies have demonstrated that TRIF is an essential adapter protein for TLR3-mediated signaling. TRIF interacts with TLR3 through their respective TIR domains. It is believed that the TIR domain of TRIF is required for its signaling activity (10, 23, 25). To determine the functions of TRIS, we performed reporter assays. Surprisingly, the TIR-less TRIF also activated the IFN-β promoters ISRE and NF-κB, although to a lesser degree in comparison with TRIF (Fig. 2A). In the poly(I-C)-responsive 293-TLR3 cells, poly(I-C) stimulation added little to the reporter activities induced by overexpression of TRIF or TRIS (supplemental Fig. S1). It is possible that overexpression of TRIF or TRIS may overwhelmingly and directly activate the downstream signaling pathway, whereas poly(I-C) stimulation activates the pathway through ectopically expressed TLR3 and endogenous TRIF/TRIS, which is not potent in these experiments.

Alternatively, specific knockdown of TRIS by two independent RNAi plasmids (plasmids 1 and 3), which had no effects on TRIF expression (Fig. 1E), inhibited poly(I-C)-induced activation of the IFN-β promoter in 293-TLR3 cells (supplemental Fig. S2 and Fig. 2B). The TRIS RNAi-1 plasmid was used for the following experiments. We also found that knockdown of TRIS inhibited poly(I-C)-induced ISRE and NF-κB activation in 293-TLR3 cells (Fig. 2B). In similar experiments, knockdown of TRIS inhibited TLR4-mediated activation of the IFN-β promoter (supplemental Fig. S3). These results suggest that TRIS is involved in TLR3- and TLR4-mediated signaling.

Molecular Mechanisms of TRIS-mediated ISRE Activation—It is unexpected that a TIR-less variant of TRIF is required for TLR3-mediated signaling. Therefore, we next determined the molecular mechanisms of TRIS-mediated signaling. Previously, it has been demonstrated that TRIF signals IRF3 activation through the noncanonical 1xk kinase TBK1 (26). In coimmunoprecipitation experiments, TRIS interacted with TBK1 (Fig. 3A). A kinase-inactive mutant of TBK1, as well as a dominant negative mutant of IRF3, inhibited TRIS-mediated ISRE activation (Fig. 3B). In addition, TRIS activated ISRE in wild type but not TBK1−/− mouse embryonic fibroblasts (MEFs) (Fig. 3C). These data suggest that TRIS activates IRF3 through TBK1.

We next determined the domains that are required for TRIS-mediated ISRE activation. Interestingly, both the N-terminal (TRIS-N) and C-terminal (TRIS-C) domains are required for TRIS-mediated ISRE activation, whereas a TRIF truncation containing the N-terminal and TIR domains (TRIF-N) was sufficient for TRIF-mediated ISRE activation (Fig. 3D). These data suggest that the N terminus together with either the TIR domain or the C terminus of TRIF are sufficient for its ability to activate ISRE.

Because both TRIF and TRIS activate ISRE through TBK1 and they contain a shared N-terminal domain that is required...
for its ability to activate ISRE, we determined whether the N
terminus of TRIF/TRIS contains a conserved TBK1-binding
site. Using a series of N-terminal deletion mutants of TRIS, we
found that amino acids 180–200 of TRIS are required for ISRE
activation in reporter assays (Fig. 3E). An alignment of this
20-amino acid region of TRIS with the TBK1-binding regions in
**TANK and NAP1**, two proteins previously shown to interact with TBK1 (27–29), revealed that two amino acids (Gln-190 and Leu-194 in TRIS/TRIF) are conserved among the three proteins (Fig. 3f). To determine whether these two amino acids are important for the interaction of TRIS with TBK1, we mutated these conserved residues to alanines and performed coimmunoprecipitation experiments. The results indicated that substitution of Leu-194 to alanine abolishes the interaction of TRIS with TBK1, while mutation of Gln-190 to alanine has no effect on the ability of TRIS to activate NF-κB in reporter assays. Vec, empty control plasmid.

**FIGURE 3.** TRIS activates ISRE through conserved TBK1-binding motif. A, interactions between TBK1 and TRIS or TRIF. 293 cells (2 × 10⁵) were transfected with the indicated expression plasmids. Cell lysates were immunoprecipitated with control mouse IgG (lg) or anti-HA (αH). The immunoprecipitates (IP) were analyzed by immunoblot (IB) with anti-FLAG (upper panel) or anti-HA (middle panel). Expression of TRIS or TRIS-4A in the lysates was detected by immunoblot analysis with anti-FLAG (bottom panel). Ab, antibody. B, RHIM is important for the ability of TRIS to activate ISRE in reporter assays. Rel. Luc. Act., relative luciferase activity. C, both the TIR and the RHIM-containing C-terminal domains can mediate dimerization or oligomerization. Wild type and mutant TRIS were schematically shown at left. Coimmunoprecipitation results were shown at the middle and right panels. The experiments were similarly performed as in A. D, schematic presentation of a chimeric protein, TBD-TIR88, which consists of the N terminus of TRIS and the TIR domain of MyD88 (left panel). This chimeric protein activated ISRE but not NF-κB in reporter assays (middle and right panels). Vec, empty control plasmid.

**FIGURE 4.** Dimerization or oligomerization is required for TRIS- or TRIF-mediated ISRE activation. A, TRIS dimerizes or oligomerizes through its C-terminal RHIM. Wild type and mutant TRIS or TRIF are schematically shown at left. Coimmunoprecipitation results were shown at the middle and right panels. In these experiments, 293 cells (2 × 10⁵) were transfected with the indicated expression plasmids. Cell lysates were immunoprecipitated with control mouse IgG (lg) or anti-HA (αH). The immunoprecipitates (IP) were analyzed by immunoblot (IB) with anti-FLAG (upper panel) or anti-HA (middle panel). Expression of TRIS or TRIS-4A in the lysates was detected by immunoblot analysis with anti-FLAG (bottom panel). Ab, antibody. B, RHIM is important for the ability of TRIS to activate ISRE in reporter assays. Rel. Luc. Act., relative luciferase activity. C, both the TIR and the RHIM-containing C-terminal domains can mediate dimerization or oligomerization. Wild type and mutant TRIS were schematically shown at left. Coimmunoprecipitation results were shown at the middle and right panels. The experiments were similarly performed as in A. D, schematic presentation of a chimeric protein, TBD-TIR88, which consists of the N terminus of TRIS and the TIR domain of MyD88 (left panel). This chimeric protein activated ISRE but not NF-κB in reporter assays (middle and right panels). Vec, empty control plasmid.

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tion of TRIS with TBK1, although mutation of Gln-190 had no significant effect (Fig. 3F). Consistently, TRIS(L194A) but not TRIS(Q190A) completely lost its ability to activate ISRE and could act as a dominant negative mutant to inhibit poly(I-C)-induced ISRE activation in 293-TLR3 cells (Fig. 3, G and H). TRIS(L194A) could activate NF-κB to a similar level with wild type TRIS (Fig. 3I). Similar results were also observed when these mutations were introduced into TRIF (data not shown). In similar experiments, TRIS(Q190A) had little effect on poly(I-C)-induced signaling in 293-TLR3 cells (supplemental Fig. 1).

In similar experiments, TRIS(L194A) had little effect on poly(I-C) induction of TRIS with TBK1, although mutation of Gln-190 had no significant effect (Fig. 3F). Consistently, TRIS(L194A) but not TRIS(Q190A) completely lost its ability to activate ISRE and could act as a dominant negative mutant to inhibit poly(I-C)-induced ISRE activation in 293-TLR3 cells (Fig. 3, G and H). TRIS(L194A) could activate NF-κB to a similar level with wild type TRIS (Fig. 3I). Similar results were also observed when these mutations were introduced into TRIF (data not shown). In similar experiments, TRIS(Q190A) had little effect on poly(I-C)-induced signaling in 293-TLR3 cells (supplemental Fig. 1).

**RHIM-mediated Dimerization or Oligomerization Is Required for TRIS-induced ISRE Activation**—Based on the above studies, it is clear that the TBK1-binding region around Leu-194 at the N terminus is required for TRIS/TRIF-mediated ISRE activation. However, the N terminus alone is not sufficient for activating ISRE. In addition to the N terminus, activation of ISRE by TRIS or TRIF also requires either the C-terminal RHIM-containing domain or the TIR domain. Previously, it has been demonstrated that the RHIM motif of TRIF is responsible for its interaction with RIP and for its ability to activate NF-κB. However, the interaction with RIP is not required for TRIS/TRIF-mediated ISRE activation (20, 21). Previous studies have shown that RHIM motifs are responsible for the homotypic interaction between RIP and RIP3, whereas TIR domains are involved in interactions of TIR-containing proteins. Based on this information, the simplest explanation for our observations is that activation of ISRE by TRIS/TRIF requires TBK1 binding via their N terminus and homotypic dimerization or oligomerization by the C terminus or TIR domain. To test this hypothesis, we first determined whether TRIS can dimerize or oligomerize through its C-terminal RHIM motif. In transient transfection and coimmunoprecipitation experiments, TRIS could interact with itself as well as TRIF (Fig. 4A). Mutation of the four RHIM amino acids (QLGL) to alanines, designated as TRIS-4A, abolished the ability of TRIS to interact with itself or TRIF (Fig. 4A) as well as its ability to activate ISRE (Fig. 4B). These data suggest that TRIS could dimerize/oligomerize or interact with TRIF through the RHIM motif, which is also required for its ability to activate ISRE. Similarly, deletion analysis indicated that the TRIF-NT mutant was able to dimerize or oligomerize. The 'TIR' domain is required for the ability of TRIF-NT to dimerize or oligomerize (Fig. 4C) and to activate ISRE (Fig. 3D). These data suggest that either the TIR domain or the C-terminal RHIM motif can provide the dimerization or oligomerization requirement for activating ISRE by TRIS or TRIF.

To further confirm that dimerization or oligomerization is an important step to activate ISRE, we determined whether an artificial dimerization or oligomerization domain can confer the N terminus of TRIS to activate ISRE. We constructed a chimeric molecule consisting of the N terminus of TRIS and the TIR domain of MyD88, a TRIF-related protein that does not activate ISRE (Fig. 4D). Overexpression of this chimeric protein strongly activated ISRE but not NF-κB (Fig. 4D). These data suggest that TBK1 binding and dimerization or oligomerization are two required events for the ability of TRIS to activate ISRE.

**RHIM Motif of TRIS Is Required for Its Interaction with RIP and Ability to Activate NF-κB**—Similarly to TRIF, TRIS is capable of activating NF-κB (Fig. 2A). In coimmunoprecipitation experiments, TRIS interacted with RIP (Fig. 5A). Mutation of the RHIM motif of TRIS abolished its interaction with RIP (Fig. 5A), as well as its ability to activate NF-κB (Fig. 5B). Interestingly, mutation of the RHIM motif of TRIS caused marked reduction but not complete loss of its interaction with RIP as well as its ability to activate NF-κB (Fig. 5A). These findings are consistent with our previous observation that both the TIR and the C-terminal domains can interact with RIP (23). These data suggest that TRIS activates NF-κB through its
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Surprisingly, we found that TRIS forms a complex with TRIF and is required for TLR3-mediated signaling. Consistent with a role of TRIF in TLR4-mediated signaling, knockdown of TRIS inhibited TLR4-mediated activation of the IFN-β promoter, suggesting that TRIS is also involved in TLR4-mediated signaling.

TRIS was identified as a 30-kDa splice variant in Western blot analysis with an antibody raised against the N terminus of TRIF. Several lines of evidence exclude the possibility that this 30-kDa protein is a degraded fragment of TRIF. First, knockdown of full-length TRIF by RNAi did not down-regulate the expression of the 30-kDa protein. Second, GenBank™ data base analysis revealed a TRIF splice variant that encodes a TIR-less protein of ~30 kDa. Reverse transcription-PCR experiments amplified a cDNA encoding for such a splice variant of TRIF. Third, an antibody raised against the N terminus of TRIF recognized both TRIF and the 30-kDa band, whereas an antibody raised against a peptide around amino acid 219 of TRIF only recognized TRIF but not the 30-kDa band. Finally, an RNAi construct targeting the joint region of TRIS mRNA (which is not in TRIF mRNA) selectively down-regulated expression of the 30-kDa protein but not full-length TRIF. These experiments suggest that TRIS is a naturally occurring splice variant of TRIF.

TRIS is constitutively and abundantly expressed in all examined and divergent types of cell lines, including human embryonic kidney 293, epithelial HeLa cells, and T lymphoma Jurkat cells, pointing to the possibility that TRIS plays an important role in TRIF-related functions. Previous studies have demonstrated that TRIS is associated with TLR3 through their respective TIR domains; therefore, a TIR-less TRIF splice variant would be expected to act as an inhibitor of the TLR3-mediated signaling. Surprisingly, overexpression of TRIS activated ISRE, NF-κB, and the IFN-β promoter, whereas specific knockdown of TRIS by RNAi inhibited poly(I-C)-induced activation of ISRE, NF-κB, and the IFN-β promoter. These results suggest that TRIS is specifically involved in TLR3-mediated signaling.

TRIS contains an N- and a C-terminal domain. Deletion analysis identified an N-terminal region that was required for the interaction between TRIS and TBK1. This region, located at amino acids 180–200 of TRIS or TRIF, is designated as TBK1-binding region (TBR). Alignment of this TBR with those of TANK and NAP1, two proteins also binding to TBK1, revealed a conserved leucine residue (Leu-194). Mutation of this residue to alanine abolished the ability of TRIS or TRIF to activate ISRE but not NF-κB. These observations suggest that both TRIS and

DISCUSSION

TRIF is a TIR domain-containing adapter protein that is recruited to TLR3 through their respective TIR domains upon ligand binding. Gene knock-out studies have demonstrated that TRIF is required for TLR3-mediated activation of IRF3 and NF-κB as well as induction of type I IFNs. In this study, we identified a TIR-less splice variant of TRIF designated as TRIS. Against a peptide around amino acid 219 of TRIF only recognized TRIF but not the 30-kDa band. Finally, an RNAi construct targeting the joint region of TRIS mRNA (which is not in TRIF mRNA) selectively down-regulated expression of the 30-kDa protein but not full-length TRIF. These experiments suggest that TRIS is a naturally occurring splice variant of TRIF.

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RHIM-mediated interaction with RIP, whereas TRIF can activate NF-κB through either its TIR or C-terminal domain.

TRIS Is Associated with TRIF upon Poly(I-C) Stimulation—Because TRIS is involved in TLR3-mediated ISRE and NF-κB activation, we determined whether TRIS is associated with TRIF under physiological conditions. We first performed gel filtration chromatography with lysates from 293-TLR3 cells untreated or treated with poly(I-C) for 30 min. In untreated cells, TRIF was eluted in complexes of more than 670 kDa as well as fractions between 220 and 440 kDa with a peak around 400 kDa, whereas TRIS only coeluted with the high molecular weight TRIF complex (Fig. 6A). Interestingly, both TRIF and TRIS shifted to overlapping high molecular weight complexes following poly(I-C) stimulation (Fig. 6A). Endogenous coimmunoprecipitation experiments with an antibody specifically recognizing TRIF but not TRIS. The immunoprecipitates (IP) and the lysates were analyzed by immunoblots with anti-TRIF-N, which recognizes both TRIF and TRIS. Ab, antibody.

FIGURE 6. Effects of TLR3 stimulation on the association between TRIS and TRIF. A, gel filtration experiments. 293-TLR3 cells were left untreated or treated with poly(I-C) for 30 min. The cell lysates were subjected to size exclusion chromatography. Fractions were analyzed by immunoblots with anti-TRIF-N. The elution positions of the molecular size markers are indicated. NS, no stimulation. B, specific recognition of TRIF but not TRIS by the anti-TRIF(219), a rabbit polyclonal antibody raised against a peptide around amino acid 219 of TRIF. 293 cells were co-transfected with HA-tagged TRIF and TRIS. The lysates were analyzed by immunoblots with anti-TRIF(219) (left panel) or anti-HA (right panel). C, endogenous association between TRIS and TRIF. 293-TLR3 cells were left untreated or treated with poly(I-C) for 30 min as indicated. The cell lysates were immunoprecipitated with control rabbit IgG or anti-TRIF(219), a rabbit antibody which only recognizes TRIF but not TRIS. The immunoprecipitates (IP) and the lysates were analyzed by immunoblots with anti-TRIF-N, which recognizes both TRIF and TRIS. Ab, antibody.
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TRIF activate the IRF3 activation pathways through their TBR-mediated interaction with TBK1. However, overexpression of the N-terminal TBR-containing domain is not sufficient to activate ISRE. Activation of ISRE by TRIS requires its dimerization or oligomerization provided by its C-terminal RHIM-containing domain. Consistently, an artificial fusion protein between the N-terminal TBR-containing domain of TRIS/TRIF and the TIR domain of MyD88, which itself does not activate ISRE, could potently activate ISRE. These studies suggest that TRIS/TRIF-mediated ISRE activation requires both their association with TBK1 and dimerization or oligomerization.

TRIS was capable of activating NF-κB, which was mediated by its association with RIP through its C-terminal RHIM. Mutation of the RHIM to alanines abolished the ability of TRIS to interact with RIP and to activate NF-κB. TRIF could activate NF-κB through the RHIM-mediated interaction with RIP; however, TRIF could also activate NF-κB through the TIR domain-mediated interaction with TRAF6 (16, 18). These studies suggest that TRIS and TRIF activate NF-κB through overlapping and distinct mechanisms.

Gel filtration chromatography experiments suggest that under unstimulated conditions, TRIF was eluted in complexes of more than 670 kDa as well as fractions between 220 and 440 kDa with a peak around 400 kDa, whereas TRIS only coeluted with the high molecular weight TRIF complex. Stimulation of the cells with the TLR3 ligand poly(I-C) caused a shift of TRIF and TRIS to overlap higher molecular weight complexes. Endogenous coimmunoprecipitation experiments suggest that TRIF could interact with TRIS after poly(I-C) stimulation of 293-TLR3 cells. These results suggest that TRIS is associated with TRIF upon TLR3 activation.

The detailed mechanisms on the involvement of TRIS in TLR3-mediated signaling are unknown at this time. Nevertheless, the identification of a TIR-less variant of TRIF that is important for TLR3-mediated signaling will certainly challenge the traditional view on the early events of TLR3-mediated signaling.

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