TRAF6 and MEKK1 Play a Pivotal Role in the RIG-I-like Helicase Antiviral Pathway*

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Type I interferons ( IFN-α/β) are essential for immune defense against viruses and induced through the actions of the cytoplasmic helicases, RIG-I and MDA5, and their downstream adaptor molecule IPS-1. TRAF6 and the downstream kinase TAK1 have been shown to be essential for the production of proinflammatory cytokines through the TLR/MyD88/kinase TAK1 have been shown to be essential for the production of proinflammatory cytokines through the TLR/MyD88/TLR/MYD88/TRIF pathway. Although binding of TRAF6 with IPS-1 has been demonstrated, the role of the TRAF6 pathway in IFN-α/β production has not been fully understood. Here, we demonstrate that TRAF6 is critical for IFN-α/β induction in response to viral infection and intracellular double-stranded RNA, poly(I:C). Activation of NF-κB, JNK, and p38, but not IRF3, was impaired in TRAF6-deficient mouse embryo fibroblasts in response to vesicular stomatitis virus and poly(I:C). However, TAK1 was not required for IFN-β induction in this process, since normal IFN-α/β production was observed in TAK1-deficient mouse embryo fibroblasts. Instead, another MAP3K, MEKK1, was important for the activation of the IFN-β promoter in response to poly(I:C). Forced expression of MEKK1 in combination with IRF3 was sufficient for the induction of IFN-β, whereas suppression of MEKK1 expression by small interfering RNA inhibited the induction of IFN-β by poly(I:C). These data suggest that IPS-1 requires TRAF6 and MEKK1 to activate NF-κB and mitogen-activated protein kinases that are critical for the optimal induction of type I interferons.

The innate immune system serves as a first line defense against viral infection. Host antiviral responses are initiated through the recognition of viral components by PRRs, including TLRs and RIG-I (retinoic acid-inducible gene I)-like helicases (RLHs) (1–3). Upon recognition, the PRRs trigger intracellular signaling pathways that induce the production of antiviral mediators, such as type I interferons (IFN-α/β), IFN-stimulated genes, inflammatory cytokines, and chemokines, such as IP-10. The expression of type I IFNs and other antiviral proteins suppresses viral replication and facilitates the adaptive immune responses. dsRNA is one of the viral components recognized by TLR3 and RNA helicases, such as RIG-I and MDA5 (melanoma differentiation-associated protein 5). TLR3 recognizes extracellular viral dsRNA internalized into the endosomes in certain types of cells, such as DCs, whereas RIG-I and MDA5 detect intracellular viral dsRNA in various types of cells, including fibroblasts (4–7).

The viral recognition by TLR3 and RIG-I/MDA5 results in rapid induction of type I IFNs through the activation of their intracellular signaling molecules (1–3). For instance, TLR3 interacts with an adaptors molecule, TRIF (8, 9), which in turn activates two IKK family proteins, TBK1 (TANK-binding kinase-1) and IKK-β (also known as IKKe) (10). Both TBK1 and IKK-β subsequently activate a transcription factor, IRF3, resulting in the initial expression of IFN-β (11, 12). Another IRF (IFN-regulatory factor) family member, IRF7, which is induced by the initial IFN-β, elicits further induction of type I IFN genes, including IFN-α and IFN-β (13). Stimulation with TLR3 ligand also activates other transcription factors, including NF-κB and AP-1, which is thought to synergize with IRF3 to induce type I IFN genes (14, 15). On the other hand, RIG-I/MDA5 bind to...

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2 The abbreviations used are: PRR, pattern recognition receptor; TRAF, TNF receptor-associated factor; IFN, interferon; TLR, Toll-like receptor; RLH, RIG-I-like helicase; dsRNA, double-stranded RNA; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; DC, dendritic cell; TRIF, TIR domain containing adaptor-inducing IFN-β; IKK, IκB kinase; TLR, Toll-like receptor; MAP3K, MAP kinase kinase; MEF, mouse embryonic fibroblast; VSV, vesicular stomatitis virus; siRNA, small interfering RNA; HA, hemagglutinin; RANtES, regulated on activation normal T cell expressed and secreted; cDC, conventional DC; MEK, mitogen-activated protein kinase; extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; G3P, glyceroldehyde-3-phosphate dehydrogenase; RT, reverse transcription; IP-10, Interferon-γ-inducible protein 10.
intracellular RNA through the C-terminal helicase domain and initiate downstream signaling cascades through the N-terminal CARD domains (4–6). The CARD domains interact with another CARD containing molecule, IPS-1 (IFN-β promoter stimulator-1; also known as MAVS, VISA, and CARDIF) (16–19), which activates TBK1/IKK-i via TRAF3, resulting in the activation of IRF3, IRF7, and NF-κB (20, 21). Therefore, both TLR3 and RIG-I/MDA5 pathways converge at the TBK1/IKK-i kinase complex.

IPS-1 contains multiple TRAF-interacting motifs (TIMs) in the proline-rich region, which can associate with the C-terminal TRAF domain of TRAF3 (22). Furthermore, IPS-1 has been shown to interact with other TRAF family members, such as TRAF6 and its downstream MAP3K, TAK1 (transforming growth factor-β-activated kinase 1) (18). Both TRAF6 and TAK1 have been demonstrated to play a critical role in the production of proinflammatory cytokines in macrophages and DCs triggered by MyD88 (myeloid differentiation factor 88) (23). The TRAF6/TAK1 signal activates a canonical IKK complex, IKKα/β/γ, resulting in the activation of NF-κB as well as MAPK cascades leading to the activation of AP-1 (25). Although TRAF6/TAK1 has been implicated in proinflammatory cytokine production induced by TLR ligands, the involvement of these molecules in the regulation of type I IFN production induced by the RLH pathway is largely unknown. In this report, we show that MEFs lacking TRAF6 exhibit a defect in the production of IFN-α/β in response to VSV infection and dsRNA, poly(I:C). On the contrary, MEFs lacking TAK1 showed normal induction of IFN-α/β. We also show that another MAP3K, MEKK1, instead of TAK1 is involved in the expression of IFN-β upon dsRNA stimulation. Both TRAF6 and MEKK1 are required for NF-κB activation but not for IRF3 activation, implicating the requirement of these molecules for the optimal induction of type I IFN in the cytosolic viral recognition systems.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Wild-type and TRAF6-deficient MEFs were prepared from day 13.5–14.5 embryos, as described previously (24). TAK1-deficient MEFs have been described previously (23). MEFs and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Cell Culture Technologies, Switzerland), penicillin, streptomycin, and glutamine (Nacalai Tesque, Japan). Conventional DCs were generated from the spleen of 2-week-old wild-type or TRAF6 knockout mice, as described previously (24). Briefly, erythrocyte-removed splenic cells were cultured in RPMI 1640 containing 10% fetal bovine serum and granulocyte-macrophage colony-stimulating factor for 7 days. Less adherent cells were collected by gentle pipetting and used as DCs. All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Kyushu University (Fukuoka, Japan). Poly(I:C) was purchased from Sigma. The anti-FLAG, M2 (Sigma), anti-T7 (Novagen), anti-HA, HA.11 (Covance), anti-IRF3 (Zymed Laboratories Inc.), and anti-p63 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used. The anti-phospho-IκBα, anti-phospho-p38, anti-phospho-JNK, anti-IκBα, anti-p38, and anti-JNK antibodies were purchased from Cell Signaling Technology. IKK-2 Inhibitor VI, U0126, SP60025, and SB203580 were purchased from Calbiochem.

Plasmids—The expression vectors for FLAG-IPS-1, FLAG-RIG-I, and IFN-β-luciferase reporter plasmid have been described (16). T7-IPS-1 and mutant RIG-I (FLAG-RIG-IN) were subcloned into T7 vectors. The T7-tagged MEKK3 expression vector T7-MEKK3, T7-Lys(391)-Met MEKK3 mutant (kn-MEKK3), and T7-Lys(432)-Met MEKK1 mutant (kn-MEKK1) were generated by PCR (26). The expression vectors for FLAG-TRAF, HA-TAK1, HA-TAB-1, FLAG-IRF3, and NF-κB-Luc have been described previously (16, 25).

Western Blotting—Cells were lysed in 0.5% Triton X-100 extraction buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl2, 2 mM dithiothreitol supplemented with phosphatase and protease inhibitor mixture (Nacalai Tesque). The debris was removed by centrifugation at 14,500 rpm for 10 min at 4 °C. The cell lysates were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG (Jackson ImmunoResearch Laboratories), using Chemi-Lumi One L Western blotting detection reagents (Nacalai Tesque). For native PAGE assay, MEFs were seeded at 4 × 105 cells/well in 6-well plates. 24 h later, cells were stimulated with 10 μg/ml of poly(I:C) using FuGENE HD for 3 h and then harvested. The cells were lysed with native PAGE sample buffer (62.5 mM Tris-Cl, pH 6.8, 15% glycerol). The extracts were separated on a 7.5% native PAGE and then immunoblotted with anti-IRF3 antibody.

RT-PCR and Real Time PCR—Total RNA was extracted from MEFs using the RNA-isol (Takara) according to the manufacturer’s protocol, and cDNA was synthesized from 1 μg of total RNA with the High Capacity cDNA reverse transcription kit (Applied BioSystems, CA). The cDNA was used as a template for PCR using KOD-plus DNA polymerase (TOYOBO, Japan) or real time PCR using the SYBR Green system (Applied BioSystems) according to the manufacturer’s protocol. Gene-specific primer sequences are as follows: G3PDH, 5′-ACAGTCATGGCCATCAC-3′ and 5′-TCCACCCGCTGTGCTGA-3′; IFN-α, 5′-ATGGCCTGCCCCTGGTTACTG-3′ and 5′-TCTTGGCTGACACCTCCAGG-3′; IFN-β, 5′-TCCAAGAAAGGACGAACATTCG-3′ and 5′-TGAGGTACATCTCCCCGTCA-3′; IP-10, 5′-GTTTGGAGATCATTGGCACCAG-3′ and 5′-GCTTACAGTACAGAGCTA-3′; RANTES, 5′-CCCTCACCATCATCCTCATC-3′ and 5′-CCCTTCCAGTGACAAACAGCA-3′.

Luciferase Reporter Assay—For the reporter gene assays, HEK293T cells were seeded at 1 × 105 cells/well in 12-well plates, or MEFs were seeded at 1 × 105 cells/well in 6-well plates. After 24 h, cells were transfected with a reporter plasmid and expression plasmids as indicated, using polyethyleneimine or FuGENE HD (Roche Applied Science). After 24 h, the cells were lysed, and luciferase activity was measured by using the luciferase assay system and chemiluminescent reagents (Pro-
FIGURE 1. **TRAF6** is required for antiviral innate immune response. **A**, wild-type (WT) or TRAF6-deficient (TRAF6KO) MEFs were infected with VSV (10 plaque-forming units/cell) for 12 h in the presence or absence of IFN-β. Cells were photographed under a microscope. Early apoptotic cells and dead cells were stained by FITC-Annexin V and detected by a flow cytometer.

**B and C**, wild-type or TRAF6-deficient cDCs (B) and MEFs (C) were infected with VSV at the indicated multiplicity of infection for 9 h or transfected with 10 μg/ml poly(I:C) for 3 h. Total RNA was extracted to analyze the expression of mRNA for IFN-α, IFN-β, IP-10, and G3PDH (loading control) by RT-PCR. Results are representative of at least three independent experiments using different MEF clones derived from four different embryos.
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mega). A plasmid containing the β-galactosidase gene was used to normalize for transfection efficiency.

Detection of Cell Death—MEFs were seeded at 5 × 10⁴ cells/well in a 24-well plate 1 day prior to treatment. The cells were left untreated or infected with VSV at the indicated multiplicity of infection. After 12 h, cells were trypsinized and collected with the supernatants, and the cell viability was determined by propidium iodide (Sigma) and Annexin V (Sigma) staining using a flow cytometer (FACSCalibur; BD Biosciences).

Confocal Microscopy—MEFs were seeded on a glass coverslip 1 day prior to treatment. The cells were transfected with 10 μg/ml poly(I:C) for 3 h, fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in PBS. After incubation in blocking buffer (1% bovine serum albumin in PBS) for 5 min, the cells were incubated with anti-IRF3 and anti-p65 antibodies, followed by ALEXA-488 and ALEXA-546-conjugated secondary antibodies (Molecular Probes). The cells were observed under a confocal laser microscope (LSM510 META; Carl Zeiss).

Virus Preparation—VSV was propagated with vero cells. The titration of VSV was determined by plaque assay using vero cells.

RNA Interference—For siRNA experiments, double-stranded RNA duplexes composed of 21-nucleotide sense and antisense oligonucleotides were synthesized. RNA oligonucleotides used for targeting mouse MEKK1 and mouse MEKK3 in this study were as follows: MEKK1#1 sense, 5’-GUAGCGAGCUGUGAUAUdTd-3’; MEKK1#2 sense, 5’-GCUGUUAAUGCUCUAGAAAdTdT-3’; MEK3 sense, 5’-GGAGGUGA-GUGGCUCUAGAgdTdT-3’. MEFs were seeded at 2 × 10⁵ cells/well in 6-well plates 24 h prior to transfection. Lipofectamine RNAiMAX (Invitrogen) was used to transfect MEFs with 40 nm siRNA, according to the manufacturerʼs instructions. 24 h after transfection, knockdown efficiency of the transfected siRNA on MEKK1 and MEKK3 mRNA expression was confirmed by RT-PCR. The primers were as follows: MEKK3, 5’-ACCTGAGCGACACGACG-3’ and 5’-TTCGGGAAATGTTCCTTCTG-3’; MEKK1, 5’-GGGAACACC-ATCCAGAAGT-3’ and 5’-CTCCTGGATCTCTTG-GCT-3’.

RESULTS

TRAF6 Is Required for Type I IFN Production Induced by Intracellular dsRNA—TRAF6 is an essential adaptor protein for production of proinflammatory cytokines, such as IL-6 and IL-12, in macrophages and DCs in response to TLR ligands (24). However, the role of TRAF6 in antiviral immunity and type I IFN production has not been clarified. To address this issue, we first infected TRAF6-deficient MEFs with the IFN-sensitive prototypic rhabdovirus, VSV. TRAF6-deficient MEFs were more vulnerable to virus infection than wild-type MEFs. Virus-induced cytolsis and Annexin V-positive fraction, including early apoptotic cells and dead cells, were markedly increased after 12-h infection in TRAF6-deficient MEFs compared with wild-type cells (Fig. 1A). This was probably due to reduced IFN production in TRAF6-deficient MEFs, because the enhanced cell death of the mutant MEFs was rescued by adding exogenous IFN-β (Fig. 1A). To investigate the role of TRAF6 in type I IFN induction, MEFs as well as conventional DCs (cDCs) from wild-type and TRAF6-deficient mice were infected with VSV. Induction of IFN-α, IFN-β, and IP-10 was almost completely diminished in TRAF6-deficient cDCs (Fig. 1B) and remarkably reduced in TRAF6-deficient MEFs (Fig. 1C, left). To mimic intracellular dsRNA generated during viral replication, we also transfected synthetic dsRNA, poly(I:C), into MEFs, using a lip-id-based transfection reagent, FuGENE HD. Up-regulation of IFN-α/β mRNA, but not IP-10 mRNA, was also severely impaired in TRAF6-deficient MEFs in response to poly(I:C) (Fig. 1C, right). These data suggest that TRAF6 is required for type I IFN production induced by intracellular dsRNA.

TRAF6 Is Involved in the RLH/IPS-1 Pathway to Activate the IFN-β Promoter—To understand the molecular mechanism by which TRAF6 induces type I IFNs in the context of intracellular dsRNA recognition, we first transfected wild-type MEFs with poly(I:C) using FuGENE HD and then examined the effect of inhibitors of various signaling molecules downstream of TRAF6 on IFN-β production (Fig. 2A). The up-regulation of mRNA expression for IFN-β and IP-10 induced by poly(I:C) was strongly suppressed by inhibitors of IKK (IKK-2 inhibitor VI, JNK (SP60025), and p38 (SB203580) but not by an inhibitor of MEK (upstream of ERK) (U0126), suggesting that the activation of IKK, p38 and JNK pathways is required for IFN-β expression. Next we transfected HEK293T cells with IRF3, TRAF6, or both constructs along with an IFN-β-promoter-luciferase reporter gene (Fig. 2B). Although overexpression of TRAF6 can activate both IKK and MAPK pathways required for IFN-β expression, the IFN-β promoter was only marginally activated by TRAF6 overexpression. However, cotransfection of TRAF6 and IRF3 exhibited a strong activation of the pro-
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moter, indicating that TRAF6 and IRF3 cooperatively activate the IFN-β promoter. Taken together, these results support the notion that the optimal activation of IFN-β promoter by intracellular dsRNA requires the coordinate activation of transcription factors IRF3, NF-κB, and ATF-2/c-Jun (14, 15).

Next we investigated whether TRAF6 functions at the downstream of the RIG-1/IPS-1 pathway. Overexpression of either RIG-IN (constitutively active form of RIG-1) or IPS-1 activated IFN-β promoter in HEK293T cells, whereas this was strongly suppressed by forced expression of the dominant negative form of TRAF6 (DN-TRAF6) in a dose-dependent manner (Fig. 2C, left). Similarly, DN-TRAF6 suppressed NF-κB activation induced by RIG-IN or IPS-1 (Fig. 2C, right). To ensure the role of TRAF6 in the RLH-mediated pathway, we further examined the IFN-β reporter assay in TRAF6-deficient MEFs. As shown in Fig. 2D, the activation of IFN-β promoter as well as NF-κB induced by forced expression of IPS-1 or transfection with poly(I:C) was severely impaired in TRAF6-deficient cells. These results suggest that RLH/IPS-1 pathway activates NF-κB via TRAF6, which is essential for the full activation of the IFN-β promoter.

To further dissect the signaling pathways downstream of TRAF6, we assessed the activation and nuclear translocation of IRF3. As shown in Fig. 2E, the homodimerization of IRF3, reflecting the activation of the molecule, was induced by poly(I:C) in MEFs even in the absence of TRAF6. To observe the nuclear translocation of IRF3 as well as NF-κB subunit, p65, we stimulated wild-type and TRAF6-deficient MEFs with poly(I:C) along with FuGENE HD and then stained the cells with anti-IRF3 and anti-p65 antibodies (Fig. 2F). Three hours after stimulation, ~30% of knock-out MEFs exhibited nuclear translocation of IRF3, which was comparable with that of wild-type MEFs. These results indicate that the activation and nuclear translocation of IRF3 are independent of TRAF6. However, nuclear translocation of p65 was impaired in TRAF6-deficient MEFs. Although all of the wild-type MEFs exhibited co-localization of p65 and IRF3 in the nucleus, more than 50% of TRAF6-deficient MEFs showed cytoplasmic retention of p65 but nuclear translocation of IRF3 (Fig. 2F). These data are consistent with previous results showing a defective NF-κB activation in the absence of functional TRAF6 in the reporter assay (Fig. 2, C and D). Moreover, poly(I:C)-induced phosphorylation of JNK and p38 was severely impaired in TRAF6-deficient MEFs (Fig. 2G). Taken together, TRAF6 appears to contribute to the activation of NF-κB and MAPK pathways rather than IRF3 activation in the RLH/IPS-1 signaling pathway in MEFs.

TAK1 Is Not Essential for RLH/IPS-1-mediated IFN-β Induction—TAK1 is essential for the TRAF6-dependent activation of NF-κB and MAPKs in Toll-like/IL-1 receptor signaling (23). Hence, we next examined whether TAK1 is involved in the RLH/IPS-1 signaling pathway. First we analyzed the ability of wild-type and TAK1-deficient MEFs to induce type I IFNs in response to VSV infection and poly(I:C) transfection. As shown in Fig. 3A, the induction of IFN-α/β in TAK1-deficient MEFs was comparable with that in wild-type MEFs. IFN-β promoter reporter activity enhanced by IPS-1 or poly(I:C) was not impaired in TAK1-deficient MEFs (Fig. 3B, left). NF-κB activity was also normally elevated by forced expression of IPS-1 in TAK1-deficient MEFs (Fig. 3B, middle), whereas lipopolysaccharide stimulation failed to up-regulate the NF-κB activity in the knock-out cells (Fig. 3B, right). The dimerization of IRF3 induced by poly(I:C) was also not affected by TAK1 deficiency (Fig. 3C). When we examined the nuclear translocation of p65 and IRF3, co-localization of both transcription factors in the nuclei was observed in both wild-type and TAK1-deficient MEFs at the same ratio (Fig. 3D). These results suggest that TAK1 is not required for type I IFN induction in the RLH/IPS-1-mediated signaling pathway.

MEKK1 Is a Candidate MAP3K to Induce IFN-β in the RLH/IPS-1 Pathway—Since TAK1 was not necessary for type I IFN induction in the RLH/IPS-1 pathway, we sought a responsible MAP3K involved in the IPS-1/TRAF6-mediated signal. MEKK1 (MEK kinase-1) and MEKK3 have been implicated in the activation of IKK and MAPKKK, leading to the activation of NF-κB and JNK (27). To examine whether MEKK1 or MEKK3 is involved in the RIG-1/IPS-1 pathway, we performed an IFN-β and NF-κB reporter assay using kinase-inactive mutants of MEKK1 (knMEKK1) and MEKK3 (knMEKK3). As shown in Fig. 4A, IPS-1- and RIG-IN-induced IFN-β promoter and NF-κB activities were inhibited efficiently by knMEKK1 and to a lesser extent by knMEKK3. Next we examined the effect of overexpression of MEKK1 and MEKK3. The forced expression of IRF3, MEKK1, or MEKK3 alone was not sufficient for the activation of the IFN-β promoter; however, cotransfection of IRF3 and MEKK1, but not MEKK3 synergistically activated the promoter activity (Fig. 4B). In order to confirm the significance of endogenous MEKK1 and MEKK3, we performed siRNA-mediated knockdown experiments. Wild-type MEFs were transfected with scrambled control, MEKK1, or MEKK3 siRNA construct, and then endogenous gene expression of MEKK1 as well as MEKK3 was sufficiently suppressed (Fig. 4C). The knockdown of MEKK1, but not MEKK3, resulted in a severe reduction of IFN-β mRNA expression induced by poly(I:C) (Fig. 4C). Induction of mRNA for IP-10 was slightly suppressed in the MEKK1 knockdown MEFs. Significant reduction of IFN-α and IFN-β levels by knockdown of MEKK1 with different siRNA oligonucleotides was also confirmed by real time PCR (Fig. 4D).

Furthermore, the suppression of type I IFNs by siRNA for MEKK1 was reverted by forced expression of rat MEKK1 (Fig. 4E). These results indicate that endogenous MEKK1 but not MEKK3 is required for type I IFN production induced by cytoplasmic dsRNA.

DISCUSSION

Recent studies have shown that the RLH family of intracellular receptors detect viral nucleic acid and signal through IPS-1 (also known as MAVS, CARDIF, and VISA) during viral infection (1–3). IPS-1 recruits TRAF3, which in turn activates TBK1 and IKK-δ, leading to the activation of IRF3 (22). IPS-1 also activates NF-κB via the FADD/RIP1 pathway mediated by caspase-8 and -10 (16, 28). In this study, we have found that TRAF6 and MEKK1 were involved in the downstream pathway of IPS-1, which facilitates NF-κB and MAPKs to enhance the type I IFN promoter. It has been shown that there are two distinct types of TIM; one is the PXEXX(acidic or aromatic residue) motif for TRAF6, and the other is the PXQX(T/S)
motif for other TRAFs, such as TRAF2 and TRAF3 (29). IPS-1 contains multiple TIMs, and TRAF1, -2, -3, -5, and -6 can interact with IPS-1 (data not shown) (see Refs. 18 and 22). Furthermore, TRAF3 is required for the activation of IRF3 but not NF-κB (21), whereas TRAF6 is required for the activation of NF-κB and MAPK but not IRF3. Thus, the IPS-1-TRAF complex is a divergent point of the RLH/IPS-1-mediated signal (Fig. 5). A similar case of the signal divergence regulated by TRAF6 and TRAF3 in the context of MyD88-dependent signaling pathway has been reported (20).

FIGURE 3. TAK1 is not essential for the RLH/IPS-1-mediated signaling pathway. A, wild-type (WT) or TAK1-deficient MEFs (TAK1KO) were infected with VSV (10 plaque-forming units/cell) for 9 h or transfected with 10 μg/ml of poly(I:C) for the indicated periods. Total RNA was extracted to analyze the expression of IFN-α, IFN-β, and G3PDH by RT-PCR. B, wild-type or TAK1-deficient MEFs were transiently transfected with either IPS-1 or empty vector along with IFN-β promoter (left) or NF-κB (middle and right) reporter plasmid and β-galactosidase. Cells were retransfected with poly(I:C) as in Fig. 2D (left) or stimulated with lipopolysaccharide (1 μg/ml) for the last 6 h of the incubation (right). 42 h after initial transfection, luciferase activity was measured and normalized based on β-galactosidase activity. C, wild-type or TAK1-deficient MEFs were stimulated with 10 μg/ml poly(I:C) for 3 h. Then cell extracts were prepared and subjected to native PAGE. Monomeric and dimeric forms of IRF3 were detected by Western blotting. D, wild-type or TAK1-deficient MEFs were stained and visualized as in Fig. 2F. A–D, representative data from three independent experiments is shown.
IKK and MAPK activities result from the activation of upstream MAP3Ks, such as TAK1, MEKK1, MEKK3, and NIK. In viral infection or dsRNA stimulation, TAK1-deficient cells normally induced type I IFN, and we could not detect the activation of TAK1 in wild-type cells in response to dsRNA (data not shown). Our findings suggest that TAK1 is not very necessary for virus-induced type I IFN production. The TAK1-independent mechanism should be controlled by TRAF6 in the RLH/IPS-1 signal pathway. Thus, we have made an attempt to identify the responsible MAP3K. TRAF6-interacting protein, ECSIT, has been shown to mediate the activation of NF-κB and JNK in TLR pathway (30). ECSIT binds to and activates MEKK1, which in turn activates IKK. MEKK3 has also been implicated in the activation of IKK and MAPKK, leading to the activation of NF-κB and JNK. For instance, the activation of NF-κB and JNK by TLR8 is TAK1-independent but MEKK3-dependent (31). Thus, it might be possible that MEKK1 and/or MEKK3 is involved in IPS-1/TRAF6-mediated IKK and MAPK activation. Our results indicate that MEKK1 is involved in the activation of the IFN-β promoter in the RLH/IPS-1-mediated anti-viral signal pathway. Forced expression of MEKK1 and IRF3 cooperatively activated the IFN-β promoter, whereas knockdown of MEKK1 resulted in the reduction of IFN-β production. The knMEKK1 suppressed the NF-κB activation. Therefore, although the precise mechanism currently remains unclear, MEKK1 seems to be necessary for the activation of IPS-1/TRAF6 pathway. Further analysis is necessary to understand how MEKK1, but not TAK1, is activated during viral infection. IPS-1 localizes in the outer mitochondrial membrane; thus, one possibility is that the localization of IPS-1 may induce the downstream kinase differently from that in TLR pathway.

IPS-1-deficient mice were found to be defective in the production of type I IFNs in response to all RNA viruses recognized by either RIG-I or MDA5, suggesting a central role in the production of type I IFNs upon viral infection (32, 33). IRF3 and IRF7 were also shown to be indispensable for type I IFN production (34, 35). On the other hand, we found that TRAF6-deficient MEFs did not completely lose the ability to induce type I IFNs, probably due to partial NF-κB activation by other mechanisms. It has been reported that IPS-1 interacts with both FADD and RIP1 (16). Both FADD and RIP1 have been reported to be critical for the induction of type I IFNs in response to poly(I:C) (36). Thus, we speculate that the absence of TRAF6 in the RLH/IPS-1 pathway is compensated for by the signal mediated by FADD and RIP1 to induce type I IFNs. Further investigation is necessary to clarify this point.

dsRNA is also recognized by TLR3 in certain cell types, and TRAF6 has been shown to be involved in the pathway depend-
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FIGURE 5. A model for TRAF6- and MEKK1-mediated signal for IFN-β production in the RLH/IPS-1 signaling pathway. Upon recognition of intracellular dsRNA by RIG-I or MDA5, RLHs recruit IPS-1 through CARD domains, which induces interaction between IPS-1 and TRAF3, TRAF6, FADD, and RIP1. TRAF3 activates TBK1/IKK-i, leading to the activation of IRF3, whereas TRAF6 may interact with MEKK1, which in turn contributes to the activation of IKK and MAPKK, subsequently activates NF-κB and AP-1, respectively. These transcription factors cooperatively activate the IFN-β expression.
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