MIP-T3 Is a Negative Regulator of Innate Type I IFN Response

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*J Immunol* published online 11 November 2011
http://www.jimmunol.org/content/early/2011/11/11/jimmunol.1100719
MIP-T3 Is a Negative Regulator of Innate Type I IFN Response

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TNFR-associated factor (TRAF) 3 is an important adaptor that transmits upstream activation signals to protein kinases that phosphorylate transcription factors to induce the production of type I IFNs, the important effectors in innate antiviral immune response. MIP-T3 interacts specifically with TRAF3, but its function in innate IFN response remains unclear. In this study, we demonstrated a negative regulatory role of MIP-T3 in type I IFN production. Overexpression of MIP-T3 inhibited RIG-I, MDA5, VISA, TBK1, and IKKe-induced transcriptional activity mediated by IFN-stimulated response elements and IFN-β promoter. MIP-T3 interacted with TRAF3 and perturbed in a dose-dependent manner the formation of functional complexes of TRAF3 with VISA, TBK1, IKKe, and IFN regulatory factor 3. Consistent with this finding, retinoic acid-inducible gene I- and TBK1-induced phosphorylation of IFN regulatory factor 3 was significantly diminished when MIP-T3 was overexpressed. Depletion of MIP-T3 facilitated Sendai virus-induced activation of IFN production and attenuated the replication of vesicular stomatitis virus. In addition, MIP-T3 was found to be dissociated from TRAF3 during the course of Sendai virus infection. Our findings suggest that MIP-T3 functions as a negative regulator of innate IFN response by preventing TRAF3 from forming protein complexes with critical downstream transducers and effectors. The Journal of Immunology, 2011, 187: 000–000.

Microbial infections are the most common cause of death in humans. While long-term protection from pathogens can be achieved by adaptive immune response, pathogens are sensed by pathogen recognition receptors (PRRs) during the early phase of infection. Each PRR recognizes relatively conserved structural motifs that are commonly found in a subgroup of pathogens (1). Activation of PRRs in turn triggers the production of cytokines including IFNs, which constitute innate immunity and facilitate the activation of adaptive response. TNF receptor-associated factor (TRAF) 3 was initially found to be associated with the cytoplasmic tails of CD40 (2, 3), but recent studies revealed its critical roles in type I IFN production (4, 5). By interacting with various partners, TRAF3 adapts activation signals from upstream PRRs, including both TLRs and RIG-I–like receptors to downstream kinases TBK1 and IKKe that phosphorylate transcription factors IFN regulatory factor (IRF) 3 and IRF7 (6, 7). TRAF3 acts as a ubiquitin ligase for itself and other substrates (6–8). In response to different upstream signals, it also undergoes K48- and K63-linked ubiquitination that is modulated by other ubiquitin ligases and deubiquitinases (8–12). In line with its positive regulatory role in antiviral response, TRAF3 signaling complexes are targeted by various viral proteins as countermeasures to inhibit IFN production (13–15); however, it is not fully understood how the adaptor function of TRAF3 in innate IFN response is achieved and regulated in the cell.

One way to derive insight into the roles and regulation of TRAF3 is to identify and characterize its interacting partners. As an adaptor, TRAF3 is known to interact with both upstream inducers and downstream effectors, including MYD88, TRIF, RIG-I, MDA5, TANK, TBK1, IKKe, and IRF3 (6, 7, 15). Other cellular and viral interacting partners of TRAF3 have also been identified (16). Among cellular TRAF3-binding proteins, MIP-T3 is one of only a few proteins that specifically interact with TRAF3 but not other TRAF proteins (17). MIP-T3 interacts constitutively with TRAF3 and microtubules through a C-terminal coiled-coil region and a central microtubule interacting domain, respectively (17). MIP-T3 has been found to localize to the cytoplasm, the primary cilium, and the centrosome (17–21). Although MIP-T3 is a component of the intraflagellar transport subcomplex B that plays an important role in primary cilium formation (18–22), nonciliary functions of MIP-T3, exemplified by the recruitment of schizophrenia susceptibility factor DISC1 to the centrosome and an inhibitory role in IL-13 signaling, have also been implicated (23–25). The biologic relevance of the interaction between TRAF3 and MIP-T3 remains elusive. Particularly, it is unclear whether MIP-T3 might modulate type I IFN production through its direct interaction with TRAF3. We therefore investigated the influence of MIP-T3 expression on TRAF3-mediated activation of IFN-β promoter activity. We demonstrated MIP-T3 to be a negative regulator of type I IFN-dependent innate immune response. We further suggested a mechanism through which...
MIP-T3 binds to TRAF3 to impede its formation of functional complexes with downstream transducers and effectors including VISA, TBK1, IKKe, and IRF3. Finally, the function of MIP-T3 was characterized in the context of viral infection. Our findings might have implications in viral pathogenesis and the design of new antivirals.

FIGURE 1. MIP-T3 inhibits RIG-I/MDA5- and TBK1/IKKε-induced activation of ISRE and IFN-β promoter activity. Constant amounts of ISRE-Luc (A) or IFN-β–Luc (B) reporter plasmid were cotransfected with a fixed amount of activator plasmid and increasing amounts of Flag–MIP-T3 plasmid into HEK293 cells. The total amount of plasmids transfected was normalized with corresponding empty vectors. pRL-CMV was used as an internal control for transfection efficiency. Expression of MIP-T3 and other stimulatory proteins was verified by Western blotting (see inset in A for an example of Western blot analysis of MIP-T3 expression. *MIP-T3; #1, \( p = 0.010598 \) by Student \( t \) test; #2, \( p = 0.01745 \) by Student \( t \) test; #3, \( p = 0.018214 \) by Student \( t \) test; #4, \( p = 0.000028 \) by Student \( t \) test. C, Effect of MIP-T3 expression on activation of human T cell leukemia virus I long terminal repeats by viral trans-activator Tax. D, Effect of MIP-T3 on tunicamycin-induced activation of GRP78 promoter. Tunicamycin was added 12 h prior to cell harvest. E, Effect of MIP-T3 on RIG-I–induced activation of NF-κB. F, Effect of MIP-T3 on poly-IC–induced activation of TLR3. HEK293 cells were transfected with ISRE-Luc, TLR3 expression plasmid, and increasing amounts of expression plasmid for NS1-V5His or Flag-MIP-T3. Forty-eight hours after transfection, cells were stimulated with 1 \( \mu \)g/ml poly-IC for another 8 h. No ISRE-Luc activity was detected in the absence of TLR3 (data not shown). Results are representative of three independent experiments. Error bars indicate SD.
Materials and Methods

Cell culture and reagents

HEK293, HEK293T, and Vero cells were obtained from the American Type Culture Collection. Primary mouse embryonic fibroblasts (MEFs) were generated from E13 embryos of wild-type mouse in C57BL/6j background. Cells were maintained in DMEM with 10% FBS (Life Technologies) and antibiotics. Cells were grown at 37°C in a humidified atmosphere with 5% CO2. Transfection of plasmid DNA was performed using Genejuice (Novagen) as described previously (15, 26). Tunicamycin was purchased from Calbiochem. Recombinant IκBα was bought from Santa Cruz. Recombinant IRF3 fused to glutathione S transferase (GST) was obtained from Abnova. Sendai virus (Cantell strain) was obtained from the American Type Culture Collection. Recombinant vesicular stomatitis virus expressing GFP (vesicular stomatitis virus [VSV]-GFP) was a gift from Dr. Jacques Perrault (San Diego State University, San Diego, CA) and Dr. Brian Lichty (McMaster University, ON, Canada) (27, 28). Viral infection of HEK293 cells and MEFs was performed as described (29).

Plasmids

cDNA clones for MIP-T3, VISA, TANK, IKKe were purchased from imaGenes (Berlin, Germany). Flag-tagged MIP-T3 was created by cloning MIP-T3 cDNA into pCMV-Tag2B (Stratagenes) using PCR amplification. It was further subcloned into pcDNA-3.1+ (Invitrogen) with an N-terminal His-V5 tag. TRAF3 was provided by Dr. Liusheng He and Dr. Peter Lipsky (National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD) (30). pIFNβ-Luc (p125-Luc) and Flag-RIG-I plasmids were provided by Dr. Takashi Fujita (Kyoto University, Kyoto, Japan) (31). TBK1 and IRF3 expression plasmids were gifts from Dr. Genhong Cheng (University of Rochester, Rochester, NY) (37). Expression plasmids for human T cell leukemia virus type I oncoprotein Tax and reporter construct LTR-Luc were described previously (34, 35). Expression plasmid for human T cell leukemia virus type I oncoprotein Tax and reporter construct LTR-Luc were described previously (34, 35). Expression plasmid for GRP78-Luc was provided by Dr. Kazuhiro Mori (HSP Research Institute, Kyoto Research Park, Kyoto, Japan) (36). Expression vector for hemagglutinin-ubiquitin (HA-Ub) was a gift from Dr. Dirk Bohmann (University of Rochester, Rochester, NY) (37). Expression plasmids for TLR3 and influenza A virus NS1 were described previously (38).

Immunoprecipitation, Western blotting, reporter assay, and protein kinase assay

Coinmunoprecipitation, Western blotting, dual luciferase assay, and protein kinase assay were performed as described previously (15, 39). Mouse monoclonal anti-Flag Ab M2 (F3165) and rabbit polyclonal anti-Flag Abs (F7425) were purchased from Sigma. Mouse monoclonal anti-V5 Ab (46-0705) was purchased from Invitrogen. Mouse monoclonal anti-Myc Ab (sc-40), rabbit polyclonal anti-Myc Ab (sc-9048) and anti-MIP-T3 (C20) Abs were purchased from Santa Cruz Biotechnology. Rabbit monoclonal anti-phospho-IRF3 (S396) Ab 4D4G (4741) was obtained from Cell Signaling. For TRAF3 ubiquitination assay, 0.1% SDS was added to the immunoprecipitation buffer and wash buffer.

Primers, small interfering RNAs, and quantitative PCR

Sequences of human-specific MIP-T3 primers were 5′-TCACACACATTCTGCAATGGA-3′ (MT3-497F) and 5′-TCACTTTCTCTCAGAT-3′ (MT3-691R). Primers for amplification of GAPDH and procedures for semiquantitative RT-PCR have been described elsewhere (40, 41).

Quantitative real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems). Quantitative PCR (qPCR) primers for mouse MIP-T3 transcript are 5′-TGGAGGGCTGA CATTGCTG-3′ (forward) and 5′-ATCACCTCCG GCTTATCTTG-3′ (reverse). Primers for mouse IFN-α are 5′-CAGCTCCTCAAG AAAGGCGAAG C-3′ (forward) and 5′-GGGACGTGTA ATCTTCTGGC T-3′ (reverse). Primers for mouse GAPDH are 5′-AGTCCGGTGT GAACGGATT G-3′ (forward) and 5′- TGTAGACCAT GTAGTGGAGG TCA-3′ (reverse). qPCR reaction was performed with a StepOne Real-Time PCR System (Applied Biosystems). Target mRNA expression was quantitated with the comparative Ct method. Relative expression level of target mRNA was calculated from 2-ΔΔCt.

Sequences for siGFP are 5′-GCAAGCGAGC CCGAAGAGC at-3′ (sense) and 5′-GACUAUCGGC GGACGCUUGC CG-3′ (antisense). Sequences for si3A and si3B against human MIP-T3 are 5′-GGAGGA GAGCUC UGAAUAAta r-3′ (sense for si3A), 5′-UUUAUUUCAG CGUCUCUCUtt r-3′ (antisense for si3A), 5′-GGAUAGGCUCAA GACAt r-3′ (sense for si3B), and 5′-UGUUCUUCUUC GGGCUAAGC c-3′ (antisense for si3B). Sequences for si3C and si3D against mouse MIP-T3 are 5′-GAGUGGAGCU GCAGAUUUCUt r-3′ (sense for si3C), 5′-G

FIGURE 2. MIP-T3 interacts with TRAF3, but not with RIG-I, TANK, TBK1, or IKKe. The indicated proteins were expressed in HEK293T cells. Western blotting (WB) was performed to probe MIP-T3 in the cell lysate with anti-V5 (α-V5). Immunoprecipitation (IP) was performed with α-Flag, and the precipitates were probed with α-Flag and α-V5, respectively.

FIGURE 3. Influence of MIP-T3 on TRAF3 polyubiquitination. The indicated combinations of HA-Ub, Flag-TRAF3, and V5–MIP-T3 were coexpressed in HEK293T cells. Cell lysates were probed with anti-Flag (α-Flag) and α-V5 by Western blotting (WB). Immunoprecipitation (IP) was performed with α-Flag. HA-Ub conjugated Flag-TRAF3 was revealed by probing immunoprecipitate with α-Flag and α-HA. Lanes 3, 4, 7 and 8 served as negative controls.
AGAAAUGUCA GCCUCCAUCU t-3' (antisense for si3C), 5'-GUGCGAGCA CAAACAGAAt-3' (sense for si3D), and 5'-UUCUGUUGUGUCUCUGACAt-3' (antisense for si3D). Transfection of small interfering RNA (siRNA) into HEK293 cells was performed as described (40) using Lipofectamine 2000 according to the manufacturer’s protocol. Transfection of siRNA into MEF cells was performed using X-treme-Gene siRNA transfection reagents (Roche).

ELISA

ELISA was performed using a mouse IFN-β ELISA kit (PBL Biomedical Laboratories) and the manufacturer’s protocol.

Plaque formation assay

Vero cells were seeded in 12-well plates. When the cells grew to 100% confluence, they were infected with serial 10-fold dilutions of the virus in DMEM culture medium for with routine rocking. One hour postinfection, infection medium was removed and infected Vero cells were then overlaid with 1:1 DMEM culture medium containing 1% agarose. At 30 h post-infection, cells were fixed with 3.7% paraformaldehyde and stained with 0.05% crystal violet.

Results

MIP-T3 inhibits RIG-I/MDA5- and TBK1/IKKe-induced activation of IFN-β promoter

To test whether and how expression of MIP-T3 might affect type I IFN production, we used luciferase reporter constructs that are driven by canonical IFN-stimulated response elements (ISRE) or IFN-β promoter. These ISREs are known to be recognized by IRF3 (42, 43). As expected, luciferase activity from both ISRE-Luc (Fig. 1A) and IFNβ-Luc (Fig. 1B) reporter constructs was remarkably stimulated by RIG-I, MDA5, VISA, TBK1, IKKe, and IRF3, which are key activators of type I IFN production (44). When MIP-T3 was expressed, a significant and dose-dependent suppression of ISRE-Luc (Fig. 1A) and IFN-β-Luc (Fig. 1B) reporter activity induced by RIG-I, MDA5, VISA, TBK1, IKKe, or IRF3 was observed in HEK293 cells. For example, when the dose of MIP-T3 plasmid introduced into cells increased from 50 to 150 ng (see inset in Fig. 1B for a representative Western blot of MIP-T3), a more than 2-fold reduction of IKKε-induced ISRE-β and IFN-β promoter-driven luciferase reporter expression was seen (Fig. 1A, 1B, lower third panel). In the control experiments, MIP-T3 did not affect the basal transcriptional activity of ISRE and IFN-β promoter (Fig. 1A, 1B, upper left panel).

In addition, to exclude the possibility of a general inhibition effect induced by overexpression of MIP-T3, we showed that MIP-T3 did not inhibit Tax-induced activation of a reporter driven by human T cell leukemia virus type I long terminal repeats (Fig. 1C). Tax is a retroviral transactivator that activates transcription from viral and cellular promoters (34, 45, 46). In addition, MIP-T3 had no effect on tunicamycin-induced activation of GRP78 promoter (Fig. 1D). GRP78 resides in endoplasmic reticulum is a protein chaperone activated by endoplasmic reticulum stressors such as tunicamycin that inhibits glycosylation (36, 47). Thus, MIP-T3
is not a general repressor of cellular transcription or translation. Because both IRF3 and NF-kB are activated in innate IFN response (31, 48, 49), we asked whether MIP-T3 might also exert an inhibitory effect on NF-kB signaling. We found that MIP-T3 had no influence on RIG-I–induced NF-kB activation (Fig. 1E), suggesting that MIP-T3 acts specifically on IRF3, but not on NF-kB. However, a strong inhibitory effect of MIP-T3 was observed when we activated TLR3 with polyinosinic-polycytidylic acid (poly-IC) to boost ISRE activity (Fig. 1F). The inhibition by MIP-T3 was even more dramatic than that mediated by influenza A virus NS1 protein, a known viral antagonist of IFNs (38). Our results indicate that MIP-T3 specifically suppresses type I IFN production induced by various activators including RIG-I, MDA5, VISA, TLR3, TBK1, IKKe, and IRF3.

**Interaction of MIP-T3 with TRAF3 and influence of MIP-T3 on TRAF3 ubiquitination**

Above we showed the inhibition of RIG-I, MDA5, TBK1, and IKKe activity by MIP-T3 (Fig. 1). To investigate the cause, we asked whether MIP-T3 might physically associate with these activators in addition to TRAF3. We coexpressed MIP-T3 and RIG-I, TANK, TRAF3, TBK1, and IKKe in HEK293T cells and performed coimmunoprecipitation assay. The target proteins were efficiently expressed and successfully precipitated (Fig. 2). MIP-T3 was found only in the TRAF3-containing precipitate (Fig. 2, lane 2), but not in all other precipitates. Thus, MIP-T3 binds specifically to TRAF3, but not to RIG-I, TANK, TBK1, or IKKe. In other words, the suppressive effect of MIP-T3 on RIG-I, TBK1, or IKKe was unlikely due to direct interaction.

TRAF3 is a ubiquitin ligase, and several negative regulators of TRAF3 have been shown to modulate K63- or K48-linked poly-ubiquitination of TRAF3 (8, 10, 12). In view of this, we next investigated whether the interaction of MIP-T3 with TRAF3 might also influence TRAF3 ubiquitination. We coexpressed HA-tagged ubiquitin and Flag-tagged TRAF3 in HEK293T cells and then probed the anti-Flag immunoprecipitate with anti-Flag and anti-HA (Fig. 3). The ladder of ubiquitinated TRAF3 proteins in the precipitate was evident (Fig. 3, lane 5). However, the ubiquitination signal of TRAF3 was only slightly reduced upon expression of MIP-T3 (Fig. 3, lane 6). Therefore, MIP-T3 did not appear to affect TRAF3 ubiquitination dramatically.

**FIGURE 5.** MIP-T3 inhibits TRAF3–IKKe complex formation in a dose-dependent manner. Progressively increasing amounts of MIP-T3 plasmid were cotransfected with TRAF3 and IKKe constructs into HEK293T cells. Immunoprecipitation (IP) and Western blotting (WB) experiments were performed as in Fig. 4B.

**MIP-T3 prevents TRAF3 from complex formation with VISA, TBK1, IKKe, and IRF3**

We demonstrated that MIP-T3 could mitigate type I IFN production (Fig. 1), but was unable to counteract TRAF3 ubiquitination (Fig. 3). However, MIP-T3 specifically interacts with TRAF3, but not with other downstream transducers or effectors in IFN production (Fig. 2), which are normally associated with TRAF3 (16). These TRAF3-associated transducers and effectors include RIG-I, MDA5, VISA (50–53), TBK1, IKKe, IRF3 (4, 5), and TANK (33). The formation of protein complexes comprising TRAF3 and these downstream proteins is essential for the activation of IFN production (4, 5). Because MIP-T3 was not in the complexes that contain RIG-I, TANK, TBK1, and IKKe (Fig. 2), we asked whether MIP-T3 and these downstream proteins might be mutually exclusive in forming a complex with TRAF3. To address this question, we extensively examined the formation of TRAF3 complexes with different downstream transducers and effectors in the presence and in the absence of MIP-T3.

We found that MIP-T3 inhibited the formation of TRAF3–VISA, TRAF3–TBK1, TRAF3–IKKe, and TRAF3–IRF3 complexes (Fig. 4, A–D); neither did MIP-T3 have any influence on the interaction between RIG-I and TRAF3 or between MDA5 and TRAF3 (data not shown). Particularly, when V5–MIP-T3 was expressed,
siRNA-mediated depletion of MIP-T3 potentiated viral activation of IFN-β promoter. A, Two independent MIP-T3–targeting siRNAs (si3A and si3B) at a concentration of 50 nM were used to deplete endogenous MIP-T3 in HEK293 cells. siGFP was used as a negative control. At the 24th hour after knockdown, reporter plasmids p125-Luc and pRL-CMV were cotransfected into the cells. Twenty-four hours later, transfected cells were infected with Sendai virus (80 hemagglutinating U/ml). Cells were harvested 0 and 24 h postinfection. Results are representative of three independent experiments. Error bars represent SD. *p = 0.024 by Student t test, #p = 0.041 by Student t test. h.p.i., hours postinfection.

B and C, Verification of MIP-T3 knockdown by siRNAs. RT-PCR was performed to amplify MIP-T3 and GAPDH transcripts. Western blot analysis was performed using anti–MIP-T3 and anti–β-tubulin Abs.

D and E, RNAi depletion of MIP-T3 in primary MEFs augmented the production of IFN-β transcript. MIP-T3 knockdown in MEF cells before and after Sendai virus infection was verified. MEFs were transfected with siGFP and siRNAs targeting mouse MIP-T3 (si3C and si3D) 48 h before Sendai virus infection. Expression level of MIP-T3 mRNA was normalized with that of GAPDH transcript. Induction of IFN-β transcript in MEFs infected with Sendai virus was also analyzed. Error bars represent SD. *p = 0.028 by Student t test, #p = 0.015 by Student t test. F, Depletion of MIP-T3 augmented IFN-β production. Infection and transfection of MEFs were performed as in D and E. Production of IFN-β was analyzed by ELISA. Error bars denote SD. (Figure legend continues)
V5–TRAF3 was not detected in the Flag–TBK1 immunoprecipitate obtained with anti-Flag Ab, whereas abundant V5–TRAF3 was found to be associated with the Flag–TBK1–containing protein complex in cells that did not express V5–MIP-T3 (Fig. 4A, lane 2 compared with lane 1). Likewise, when V5–MIP-T3 was overexpressed, V5–TRAF3 was absent from the Flag–IKKe or Myc–IRF3 immune complex (Fig. 4B, 4C, lane 2 compared with lane 1), and V5–VISA was almost undetectable in the Flag–TRAF3 precipitate (Fig. 4D, lane 2 compared with lane 1). In sharp contrast, MIP-T3 appeared to have no influence on TANK–TRAF3 or IRF3–TBK1 interaction (Fig. 4E, 4F). As such, comparable amounts of V5–TRAF3 were recovered from Flag–TANK–containing immune complex in cells either not expressing or expressing V5–MIP-T3 (Fig. 4E), whereas similar levels of endogenous IRF3 were found to be associated with TBK1–Flag immunoprecipitate in V5–MIP-T3-expressing or V5–MIP-T3–nonexpressing cells (Fig. 4F). That is to say, MIP-T3 did not prevent TRAF3 from interacting with all its partners promiscuously, nor did it impede all downstream events. Instead, MIP-T3 specifically perturbs the formation of particular TRAF3 complexes.

Using TRAF3–IKKe as an example, we showed that the progressively elevated expression of MIP-T3 in HEK293T cells correlated with a gradual diminution of IKKe-associated TRAF3 (Fig. 5, lanes 2–4). Likewise, dose-dependent inhibition of TRAF3–VISA, TRAF3–TBK1, and TRAF3–IRF3 by MIP-T3 was also observed when similar experiments were performed (data not shown). Hence, one functional consequence of the interaction between MIP-T3 and TRAF3 is the perturbation of complex formation between TRAF3 and downstream proteins including VISA, TBK1, IKKe, and IRF3.

**MIP-T3 inhibits phosphorylation of IRF3 in cultured cells without affecting TBK1 kinase activity in vitro**

Above we showed that MIP-T3 inhibits type I IFN production by inhibiting TRAF3 complex formation (Fig. 4). IRF3 is one major transcription factor regulating IFN-β transcription. Phosphorylation of IRF3 is a critical event in IFN production (54). Phosphorylated IRF3 would dimerize and translocate into the nucleus to activate the IFN-β promoter (55). To shed light on the mechanism by which MIP-T3 inhibits IFN production, we next explored whether MIP-T3 might affect RIG-I–induced and TBK1–induced phosphorylation of IRF3. Phosphorylated IRF3 in cultured cells overexpressing RIG-I or TBK1 was readily detected with a phosphospecific Ab (Fig. 6A, lanes 1 and 3). Upon overexpression of MIP-T3, the level of phospho-IRF3 induced by RIG-I or TBK1 was significantly reduced to undetectable level (Fig. 6A, lanes 2 and 4). Thus, MIP-T3 plausibly perturbs phosphorylation and activation of IRF3 in cultured cells.

To investigate whether the activity of kinases that phosphorylate IRF3 is also inhibited by MIP-T3, in vitro kinase assay was performed using TRAF3 immunoprecipitated from HEK293T cells expressing increasing levels of MIP-T3 (Fig. 6B). Because TBK1 recovered from MIP-T3–expressing cells could still phosphorylate recombinant IκBα and GST–IRF3 to a similar extent as the enzyme prepared from cells that did not express MIP-T3 (Fig. 6B, lanes 1–3), the kinase activity of TBK1 is not inhibited by MIP-T3. Similar results were also obtained with another IRF3 kinase IKKe (data not shown). Thus, the perturbation of IRF3 phosphorylation by MIP-T3 is unlikely mediated by direct inhibition of IRF3 kinase activity.

**Depletion of MIP-T3 potentiates viral production of IFN-β**

The above experiments were all conducted with overexpression assays (Figs. 1–6). To verify our findings in a loss-of-function experiment, we used MIP-T3–targeting siRNAs to deplete endogenous MIP-T3 in HEK293 and MEFs (Fig. 7). To control for nonspecific effects induced by siRNA in general, an siRNA against GFP (siGFP) was also used. In addition, to minimize the nonspecific effects caused by any particular siRNA targeting MIP-T3, two independent MIP-T3–depleting siRNAs targeting different regions of MIP-T3 were used in each experiment. siRNAs-transfected cells were further challenged with Sendai virus. The activity of IFN-β–Luc reporter was significantly induced by Sendai virus (Fig. 7A, column 4 compared with column 1). Notably, this induction was further augmented in cells transfected with MIP-T3–targeting siRNAs (Fig. 7A, columns 5 and 6 compared with column 4). The augmentation observed was statistically significant (p < 0.05 by Student t test). The two independent MIP-T3–depleting siRNAs (si3B and si3A) behaved in a similar manner, lending support to the specificity of the effect. The effectiveness of MIP-T3 knockdown was verified by semiquantitative RT-PCR and Western blotting using MIP-T3 specific primers and Abs, respectively (Fig. 7B, 7C).

To gain further insight into the physiologic role of MIP-T3 in normal fibroblasts, we also depleted the expression of MIP-T3 in MEFs using two siRNAs, which specifically target mouse MIP-T3 transcript. Real-time qPCR analyses indicated that these siRNAs were highly effective in dampening the expression of MIP-T3.

\[ p = 0.00475 \text{ by Student } t \text{ test, } p = 0.0152 \text{ by Student } t \text{ test.} \]
MIP-T3 inhibits type I IFN production during the course of Sendai virus infection

The effect of MIP-T3 knockdown on viral induction of IFN was moderate (Fig. 7A, 7E, 7F). This finding led us to the hypothesis that MIP-T3 might be inactivated during the course of viral infection. To investigate this and to shed further light on the biologic relevance of MIP-T3 in the context of viral infection, we monitored the dynamic changes in the composition of the TRAF3 complex in Sendai virus-infected HEK293T cells. Interestingly, the amounts of MIP-T3 communoprecipitated with TRAF3 were gradually diminished during the course of Sendai virus infection (Fig. 8, lanes 2 and 3 compared with lane 1). Concurrently and in keeping with the essential role of TRAF3–IRF3 complex in viral activation of innate immunity, the relative amounts of both phosphorylated IRF3 and TRAF3-associated IRF3 increased with time (Fig. 8, lanes 2 and 3 compared with lane 1). Thus, the dissociation of MIP-T3 from TRAF3 accompanied by an enhanced association of IRF3 with TRAF3 and enhanced phosphorylation of IRF3 in virus-infected cells might play an important role in viral activation of IFN response.

Discussion

Type I IFNs are important components of innate immunity, which combat microbial infections at the front line (44, 56, 57). To ensure a rapid but transient response, the production of type I IFN is tightly regulated. The identification of MIP-T3 as a TRAF3 binding partner was made in 2000 (17). Although there is increasing evidence to indicate a critical role of TRAF3 in the regulation of innate type I IFN response (4, 5), how TRAF3 function is influenced by its binding proteins remains largely unknown. In this study, we provided several lines of evidence to support the conclusion that MIP-T3 functions as a negative regulator of type I IFN production. First, overexpression of MIP-T3 suppressed RIG-I/MDA5- and TBK1/IKKe-induced ISRE and IFN-β promoter activity (Fig. 1). Second, MIP-T3 interacted specifically with TRAF3 (Fig. 2) and prevented it from forming protein complexes with downstream transducer and effector proteins (Figs. 4, 5). Third, MIP-T3 inhibited RIG-I– and TBK1-induced IRF3 phosphorylation in cultured cells without affecting the IRF3 kinase activity (Fig. 6). Fourth, siRNA-mediated depletion of MIP-T3 augmented viral induction of IFN production and compromised viral replication in normal fibroblasts (Fig. 7).

Finally, MIP-T3 was dissociated from TRAF3 during the course of Sendai virus infection (Fig. 8). Negative regulators of type I IFN production have been well described (58). PIN1 and LGP2 have been suggested to downregulate type I IFN production at IRF and RIG-I–like receptor levels, respectively (59–61), although a positive regulatory role of laboratory of genetics and physiology 2 upstream of RIG-I and MDA5 has also been reported recently (62). Deubiquitinases DUBA (11) and OTUB1/2 (10) as well as ubiquitin ligases cIAP1/2 (12) and Triad3A (9) have also been shown to be negative regulators of innate immunity by modulating degradative and nondegradative polyubiquitination of TRAF3. Other deubiquitinases and ubiquitin ligases such as A20, TAX1BP1 (63, 64), CYLD (65), and RNF125 (66) have also been thought to inhibit innate immunity by targeting other transducers and effectors such as RIG-I, TBK1, and IRF3/7. In addition, a newly identified inhibitor of IFN production named optineurin is also a ubiquitin-binding protein that likely fulfills its repressive role by modulating ubiquitination (67). However, the formation of functional TRAF3-containing signaling complexes has been shown to be targeted by various viral proteins (13–15). In this study, we characterized MIP-T3 to be a cellular inhibitor of type I IFN production that impedes TRAF3 complex formation (Fig. 4). MIP-T3 is unique among cellular inhibitors of innate immunity, because it appears to affect IFN production only slightly (Fig. 3) but is capable of preventing TRAF3 from engaging downstream transducers (Fig. 4). This finding indicates that both cellular and viral inhibitors of IFN production might function by the same mechanism of modulating the formation of TRAF3 complexes. It will be of great interest to understand whether MIP-T3 competes with selected TRAF3-interacting proteins by preoccupying the same binding domains in TRAF3 with higher affinity, speed, or stability.

Although the knockdown of MIP-T3 by RNAi further enhanced viral induction of IFNs (Fig. 7), the potentiating effect was not as dramatic as in the case of other known cellular negative regulators of IFN production such as PIN1 (59), DUBA (11), and mitofusin proteins (68–70). This finding might be explained by the incomplete knockdown of MIP-T3 as seen in Fig. 7B–D. Alternatively, for viral activation of IFN production, MIP-T3 might already be inactivated. Therefore, further knockdown of MIP-T3 might not have a substantial effect on top of viral induction. Consistent with this, we demonstrated the dissociation of MIP-T3 from TRAF3 during the course of Sendai virus infection (Fig. 8), which lent further support to the notion that MIP-T3 is biologically important in viral activation of IFN production.

MIP-T3 is a ciliary protein required for ciliogenesis and intraflagellar transport (18–21). Particularly, MIP-T3 has been suggested to be a new component of intraflagellar transport subcomplex B (71). Primary cilia are ubiquitous in mammalian cells, and they fulfill important functions in cell motility in addition to cell signaling and homeostasis (72, 73). In particular, primary cilia serve as a platform for integration of the hedgehog signaling pathway and might also regulate platelet-derived growth factor receptor-α and Wnt signaling (72, 74). Thus, it will not be too surprising if the signal transduction process leading to type I IFN production might also take place at the primary cilia. Of note, some viruses such as influenza A virus and severe acute respiratory syndrome coronavirus preferentially infected apical ciliated side of the respiratory and gastrointestinal epithelia (75, 76). It is therefore intriguing to elucidate whether MIP-T3 and MIP-T3–binding proteins concentrated in the cilia might facilitate viral infection by compromising innate immunity.

Acknowledgments

We thank Drs. Dirk Bohmann, Genhong Cheng, Takashi Fujita, Dominique Garcin, Liusheng He, Brian Lichty, Peter Lipsky, Kazutoshi Mori, and Jacques Perrault for reagents and members of the Jin laboratory for critical reading of the manuscript.
Disclosures
The authors have no financial conflicts of interest.

References


