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Novel Function of Trim44 Promotes an Antiviral Response by Stabilizing VISA

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Virus-induced signaling adaptor (VISA) functions as a critical adaptor in the regulation of both the production of type I IFNs and the subsequent control of the innate antiviral response. In this study, we demonstrate that tripartite motif (Trim)44 interacts with VISA and promotes VISA-mediated antiviral responses. The overexpression of Trim44 enhances the cellular response to viral infection, whereas Trim44 knockdown yields the opposite effect. Trim44 stabilizes VISA by preventing VISA ubiquitination and degradation. These findings suggest that Trim44 functions as a positive regulator of the virus-triggered immune response by enhancing the stability of VISA.

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Materials and Methods
cDNA constructs and reagents
Human Trim44 and VISA were amplified by PCR using cDNA from SeV-infected HEK293 cells and were subsequently cloned into a pcDNA3 vector. The IFN-β luciferase reporter expression construct was a gift from Katherine A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). The NF-κB luciferase reporter was obtained from Chen Wang (Shanghai Institutes for Biological Sciences, Shanghai, China). The ISRE luciferase reporter was purchased from Stratagene. Both hemagglutinin (HA)-K48-ub and HA-K63-ub were provided by Hongbing Shu (Wuhan University, Wuhan, China). PCBP2 was a gift from Zhongfan Jiang (Peking University, Beijing, China). The other plasmids were either generated or obtained as described previously (23, 28). The anti-HA Ab was obtained from Covance (HA.11; 16B2; CO-MMS-101R), and the anti-Flag (M2; F3165) and anti-
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SPI (59809) Abs were purchased from Sigma-Aldrich. The anti-Trim44 Ab (15111-1-AP) was purchased from Proteintech Group, and the anti-IRF3 Ab (sc-9082) was obtained from Santa Cruz Biotechnology. The Ab specific for IRF3 phosphorylation at residue Ser396 (4947), the anti-p65 Ab (4764), the phospho-p65 (Ser536) Ab (3033), the anti–IκB-α Ab (9242), and the phospho–IκB-α (Ser32/36) Ab (9246) were all purchased from Cell Signaling Technology. The VISA Ab was obtained from Hongbing Shu. SeV was provided by the Wuhan Institutes of Virology, Chinese Academy of Sciences.

Cell culture and transfection

HEK293T and HEK293 cells were cultured in DMEM, and L929 cells were grown in RPMI 1640. All cells were supplemented with 10% FBS (Invitrogen), 4 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin under humidified conditions with 5% CO2 at 37˚C. Transfection of HEK293T cells and HEK293 cells was performed with either Lipofectamine (Invitrogen) or Lipofectamine 2000 (Invitrogen).

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot analysis were performed as described previously (23). In short, either HEK293T or HEK293 cells were transfected with various combinations of plasmids. At 24 h after the transfection, the cell lysates were prepared in lysis buffer and incubated with the indicated Ab together with protein A/G Plus-agarose immunoprecipitation reagent (Santa Cruz Biotechnology) at 4˚C for 3 h or overnight. After three washes, the immunoprecipitates were boiled in SDS sample buffer for 10 min and analyzed by immunoblot. For endogenous coimmunoprecipitation experiments, HEK293 cells were infected with SeV for 4 h, and the cell lysates were then prepared in lysis buffer and incubated with 0.5 μl anti-VISA or rabbit IgG. The subsequent procedures were performed as described above.

Luciferase assays

The luciferase assays were performed as described previously (28). Briefly, either HEK293T or HEK293 cells were transfected with the reporter and the indicated expression plasmids, together with the Renilla luciferase plasmids as an internal control. The total DNA concentration was kept constant by supplementing with the empty vector pcDNA3.0. At 24 h after the transfection, the cells were either lysed in passive lysis buffer or infected with SeV for an additional 24 h and then lysed. The luciferase activity of the lysates was analyzed using a dual luciferase reporter assay system (Promega).

Nuclear extracts

The nuclear extracts were prepared as described previously (29).

Real-time PCR

Total RNA was extracted from the cultured cells with TRIzol reagent (Invitrogen), and the cDNA was prepared as described previously (23). All gene transcripts were quantified by real-time PCR with SYBR Green qPCR Master Mix using a 7900HT Fast real-time PCR system (Applied Biosystems). The relative fold induction was calculated using the 2^ΔΔCt method. The primers used for real-time PCR were as follows: IFN-β, forward, 5'-AGTGTCAGAAGCTTCCAGGGTC-3', reverse, 5'-TGAGGCTAGTATCAACGCTCC-3'; TNF-α, forward, 5'-ATGGACACTGAAAGCATGACC-3', reverse, 5'-GAGGGCTTGATTAGAGAGGTC-3'; and GAPDH, forward, 5'-TGACCTGACTGACCCAG-3', reverse, 5'-GGGTGTGCTGTTGAAGTCA-3'.

RT-PCR

Total RNA was isolated from HEK293 cells using TRIzol reagent (Invitrogen) and RT-PCR analysis was performed to measure the expression levels of VISA and GAPDH. The gene-specific primer sequences were as follows: VISA, forward, 5'-ATGCTGTTTGTGGAAGACAA-3', reverse, 5'-GAACTGACTCCAGGGGCC-3'; and GAPDH, forward, 5'-GGATTGTGGCTATGGG-3', reverse, 5'-GGAAGATGGTGAGGGATT-3'.

ELISA

The levels of human IFN-β and TNF-α in the supernatants were determined using IFN-β (PBL) and TNF-α (eBioscience) ELISA kits, respectively, according to the manufacturers’ instructions.

RNA interference

Trim44-Stealth-RNA interference was designed by the Invitrogen BLOCK-iT RNAi Designer. The small interfering RNA (siRNA) sequences used were as follows: T1, forward, 5'-UCUAGACAUUCAUCCCAUAGG-3', reverse, 5'-GAAGCAAGACUCAGGGAGCUA-3'; T2, forward, 5'-AUCUCAGACUAAGACUGCGCC-3', reverse, 5'-UGCCCAACGCUCUGAGCUAGAUAG-3'; and T3, forward, 5'-AUCUGAGGGAAUGGAGUCUCC-3', reverse, 5'-GCCAGACUACC-3'.

The negative control siRNA was purchased from Invitrogen (catalog no. 12955-300). HEK293T or HEK293 cells were transfected with siRNA using Lipofectamine 2000. At 24 h after transfection, the cells were used for further experiments.

Virus manipulation

The cells were incubated in serum-free medium for 2 h, and SeV, VSV, or NDV-GFP was added to the medium at multiplicities of infection of 0.2–1 depending on the experiment. The medium was then removed, and DMEM containing 10% FBS was added.

Statistics

The data are presented as the means ± SD from at least three independent experiments. The statistical comparisons between the different treatments were performed using the unpaired Student t test, and p < 0.05 was considered statistically significant.

Results

Trim44 positively regulates virus-induced type I IFN and NF-κB signaling

To determine whether the Trim protein family plays a role in antiviral immunity, we assessed the effects of the Trim family members on the IFN-β promoter reporter activity triggered by SeV. The luciferase assay results revealed that the overexpression of Trim44 in HEK293 cells enhanced SeV-induced IFN-β activity (Fig. 1A). Trim44 protein expression was induced dramatically upon SeV infection and peaked at 8 h in HEK293 cells (Supplemental Fig. 1). To confirm that Trim44 plays a positive role in the antiviral response, additional experiments were performed in HEK293 cells. Real-time PCR and ELISA results indicated that SeV-induced IFN-β and TNF-α levels were significantly increased at both the mRNA and protein levels in the presence of Trim44 (Fig. 1B, 1C).

It has been well documented that the induction of type I IFN and proinflammatory cytokines is dependent on the activation of both IRF3 and NF-κB. Therefore, we tested the effect of Trim44 overexpression on the SeV-induced activation of IRF3 and NF-κB in HEK293 cells. The data indicated that the phosphorylation of IRF3 and IκB-α triggered by the SeV was increased when HEK293 cells were transiently transfected with Trim44 (Fig. 1D). The nuclear extracts isolated from the SeV-infected HEK293 cells confirmed that the nuclear translocation of activated IRF3 and p65 was enhanced in cells transfected with the Trim44 plasmid (Fig. 1E).

Taken together, the results suggest that Trim44 positively regulates the virus-triggered type I IFN and NF-κB signaling pathways.

Trim44 knockdown impairs virus-induced type I IFN and NF-κB signaling

To further confirm whether Trim44 is involved in the regulation of virus-induced signaling under physiological conditions, we used the knockdown approach to examine the potential role of Trim44 on type I IFN production. We designed three pairs of siRNA oligonucleotides specific for Trim44 RNA (T1, T2, and T3) and a control siRNA (SC). The results showed that T3 most efficiently inhibited Trim44 expression in HEK293 cells transfected with Trim44 and T3 could also silence endogenous Trim44 expression in untransfected HEK293 cells (Fig. 2A). T3 was then used to examine the effects of the Trim44 knockdown on the SeV-triggered immune response in HEK293 cells. In luciferase assays, the knockdown of endogenous Trim44 decreased SeV-induced IFN-β reporter activation (Fig. 2B). Consistently, Trim44 knockdown
Renilla activity was normalized to phosphorylated IRF3 and p65 were analyzed by immunoblot. Luciferase and then fractionated into cytosolic and nuclear subfractions. The total and treated cells. (SeV or left untreated. The mRNA results are relative to those of the untreated cells. (A) Identification of Trim44 as a new regulator of RIG-I signaling. (A) Luciferase activity in HEK293 cells transfected with an IFN-β luciferase reporter, together with the empty vector (Vec) or the Trim44 plasmid, and then infected with SeV for 24 h. The results are presented relative to cells without SeV infection. (B) After transfection with the indicated plasmids, HEK293 cells were either infected with SeV or left untreated and then analyzed via real-time PCR (B) or ELISA (C) assessment of IFN-β and TNF-α expression levels. The mRNA results are relative to those of uninfected cells. (D) HEK293 cells were transfected as described in (B) and stimulated with SeV for the indicated times. Total and phosphorylated IRF3 and IκB-α expression levels were assessed in the cell lysates. (E) HEK293 cells were transfected and infected as described in (D) and then fractionated into cytosolic and nuclear subfractions. The total and phosphorylated IRF3 and p65 were analyzed by immunoblot. Luciferase activity was normalized to Renilla luciferase activity. The data are representative of three independent experiments and are presented as means ± SD (n = 3). *p < 0.05, **p < 0.01.

To elucidate the molecular mechanisms responsible for the Trim44-mediated positive regulation of the SeV-induced signaling pathway, we first performed luciferase assays to identify the target of Trim44. Because SeV activates type I IFN and proinflammatory cytokine production through a signaling pathway involving RIG-I, VISA, and TBK1, the effects of Trim44 overexpression on IFN-β reporter activation mediated by these molecules were detected. As shown in Fig. 3A, RIG-I– and VISA-induced IFN-β reporter activation was significantly enhanced by Trim44 overexpression, whereas TBK1-induced IFN-β reporter activation showed no difference with or without Trim44 expression. Furthermore, the overexpression of Trim44 enhanced VISA-mediated ISRE and NF-κB reporter activity, but it failed to influence TBK1-mediated signaling (Fig. 3B). It is hypothesized that Trim44 targets VISA to enhance RIG-I signaling. To address this hypothesis, we investigated whether Trim44 interacted with VISA. HA-Trim44 and Flag-VISA were cotransfected in HEK293T cells, and coimmunoprecipitation assays revealed that Trim44 interacted with VISA (Fig. 3C, 3D). We further performed endogenous coimmunoprecipitation experiments, which indicated that Trim44 interacted with VISA in both uninfected HEK293 cells and SeV-infected HEK293 cells (Fig. 3E). We then determined which regions of the VISA protein were required for association with Trim44. Various Flag-tagged VISA mutants were expressed in HEK293T cells with HA-tagged Trim44, and the coimmunoprecipitation experiments were performed. As shown in Fig. 3F, only full-length of VISA was able to interact with Trim44. Next, we aimed to determine which part of Trim44 is essential for this interaction. Unlike most Trim family members, Trim44 does not have a RING domain. Instead, Trim44 is composed of an N-terminal zinc finger domain found in ubiquitin hydrolases (ZF UBP), a B box domain, and a coiled-coil domain in the C terminus. We found that the only internal portion of Trim44 harboring the B box domain (residues 174–215) was able to bind VISA (Fig. 3H). Taken together, these data suggest that Trim44 interacts with VISA to regulate the RIG-I signaling pathway and that the B box domain of Trim44 is required for this interaction.

Trim44 enhances the stability of VISA

We next sought to determine the mechanism by which Trim44 regulated the VISA-mediated signaling pathway by the interaction with VISA. In HEK293 cells infected with SeV, the expression of VISA diminished considerably 6 h postinfection, and this effect was inhibited with the proteasome inhibitor MG132 (Supplemental Fig. 2). It has been reported that Trim44 interacts with and stabilizes Trim17 (30). Therefore, we hypothesized that Trim44 might exert an effect on the stability of VISA. To test this hypothesis, HEK293 cells were infected with SeV after transfection with Trim44 or the control plasmid, and the cells were then analyzed by Western blot. We found that Trim44 expression enhanced the expression of VISA significantly at 12 h following SeV infection compared with control cells (Fig. 4A). To exclude the possibility that the decrease in VISA protein levels was caused by lower expression of the gene, we performed RT-PCR to analyze the same HEK293 cells expressing various genes and found that the abundance of VISA mRNA did not change (Fig. 4A). More-
over, the mutant of Trim44 lacking the B box domain, which is unable to bind with VISA, reduced the capacity to enhance the expression of VISA (Fig. 4B). Next, we addressed whether the reduction in endogenous Trim44 expression levels affected the stability of VISA. As shown in Fig. 4C, the knockdown of Trim44 decreased the expression levels of VISA after SeV infection. Moreover, Trim44 knockdown inhibited VISA-mediated IFN-β promoter activation in a luciferase reporter assay (Fig. 4D). Collectively, our data demonstrate that Trim44 enhances the stability of VISA, which suggests that Trim44 may influence VISA ubiquitination in the process.

Trim44 inhibits VISA ubiquitination

To explore how Trim44 regulated the stability of VISA, the status of VISA ubiquitination upon virus infection was assessed. Trim44 was cotransfected with VISA and HA-tagged ubiquitin, and the ubiquitination of VISA in the cells was examined following SeV infection. As expected, Trim44 expression prevented the polyubiquitination of VISA and this prevention was much more obvious in SeV-infected cells (Fig. 5A). Moreover, the polyubiquitination of VISA decreased considerably with increasing Trim44 expression levels (Fig. 5B). It has been reported that PCBP2 enhances the ubiquitination and the subsequent degradation of VISA (31), and we therefore examined the effects of Trim44 on the PCBP2-mediated degradation of VISA. Our results revealed that Trim44 dramatically inhibited the PCBP2-induced ubiquitination and degradation of VISA (Supplemental Fig. 3). Because proteasomes normally recognize and degrade proteins modified with K48-linked polyubiquitin chains, we used expression plasmids for ubiquitin mutants retaining only a single lysine residue, K48 (ubiquitin-K48) or K63 (ubiquitin-K63), to determine whether Trim44 influenced the K48- or K63-linked ubiquitination of VISA. As shown in Fig. 5C, immunoprecipitation and immunoblot analysis indicated that Trim44 inhibited VISA ubiquitination.
with ubiquitin-K48 but not with ubiquitin-K63. Additionally, Trim44 knockdown significantly increased the K48-mediated polyubiquitination of VISA (Fig. 5D). Furthermore, similar effects were observed for endogenous VISA polyubiquitination upon SeV stimulation (Fig. 5E). Taken together, these data suggest that Trim44 inhibits the K48-linked ubiquitination of VISA, which is recognized and degraded by the proteasome system.

**Trim44 positively regulates the antiviral response**

Because Trim44 prevented VISA from ubiquitination and degradation, and it enhanced the virus-induced production of IFN-β and TNF-α, Trim44 may play a role in the inhibition of viral infection. To further demonstrate this novel function of Trim44, we investigated whether Trim44 is capable of suppressing VSV infection in HEK293 cells. When Trim44 was overexpressed, the plaque assays indicated that the production of VSV was significantly reduced in HEK293 cells (Fig. 6A). Similarly, the overexpression of Trim44 significantly suppressed NDV-GFP virus replication in HEK293 cells (Fig. 6B). In contrast, Trim44 knockdown promoted NDV-GFP virus replication in the cells (Fig. 6C, 6D). These results suggest that Trim44 is a positive regulator in the control of virus infection.

**Discussion**

VISA is critical for innate antiviral immunity, as it acts as the sole adaptor for RIG-I-like helicases. However, the regulation mechanisms of VISA signaling are not well understood. In this study, we characterized the novel functions of Trim44 in the regulation of RIG-I-mediated signaling by targeting VISA. First, the exogenous expression of Trim44 enhanced the virus-induced type I IFN and NF-κB signaling pathways. This induced accumulation of IRF3 and NF-κB in the nucleus and increased the expression of IFN-β and TNF-α, whereas the knockdown of Trim44 exerted the opposite effect. Second, the reduction in endogenous Trim44 expression levels significantly sensitized cells to virus infection and resulted in a significant increase in the production of VSV or NDV-GFP, whereas the exogenous expression of TRIM44 significantly repressed NDV-GFP virus replication. Third, Trim44 interacts with VISA. Domain mapping results suggested that the B box domain of Trim44 plays an important role in the interaction with VISA. Fourth, Trim44 enhanced the stability of VISA. The expression levels of VISA decreased dramatically 6 h after SeV infection. The exogenous expression of Trim44 blocked this decrease, whereas Trim44 knockdown enhanced this decrease. Fifth, treating SeV-infected cells with MG132 resulted in an increased accumulation of VISA protein. Furthermore, VISA was polyubiquitinated after

**FIGURE 5.** Trim44 reduces the K48-linked ubiquitination of VISA. (A) Immunoblot analysis of HEK293 cells transfected with various combinations of plasmid as indicated, stimulated with SeV for 6 h, immunoprecipitated with anti-Flag, and analyzed via immunoblot with an anti-HA Ab. (B) HEK293T cells were transfected with the indicated plasmids and increasing concentrations of Trim44 (0, 0.5, and 1 μg). Immunoprecipitation and immunoblot analysis were then performed. (C) Immunoassay of HEK293T cells transfected with various combinations of plasmids, including Flag-VISA, Myc-Trim44, HA-K48-ubi, and HA-K63-ubi, followed by immunoprecipitation with anti-Flag and immunoblot with anti-HA. (D) HEK293T cells were transfected as described in (C), except that the control siRNA (SC) and the Trim44-specific siRNA (T3) were transfected instead of the Myc-Trim44 plasmid. (E) HEK293 cells were transfected with either HA-K48-ubi or HA-K63-ubi and treated with SC or T3, and the cells were then left uninfected or infected with SeV for 6 h. Immunoprecipitation and immunoblot analysis were then performed. The data are representative of three independent experiments with similar results.

**FIGURE 6.** Trim44 positively regulates antiviral responses. (A and C) HEK293 cells transfected with the empty vector (Vec), Trim44 plasmid (A), control siRNA (SC), or Trim44-specific siRNA (T3) (C) were infected with VSV. The titers of VSV were determined by standard plaque assay. The data are presented as means ± SD (n = 3). *p < 0.05, **p < 0.01. (B) NDV-GFP replication in HEK293 cells transfected with various combinations of Trim44 and VISA plasmids was measured by fluorescence microscopy. Top, GFP + cells in one field of vision; below, total cells in the same field of vision. (D) NDV-GFP replication in HEK293 cells transfected with the indicated plasmid or siRNA was measured by fluorescence microscopy. Top, GFP+ cells in one field of vision; below, total cells in the same field of vision. The data are representative of three independent experiments.
SeV infection, and this polyubiquitination was K48-linked. These data indicate that VISA undergoes proteasome-dependent degradation, for which process K48-linked polyubiquitination is essential. The K48-linked polyubiquitination of VISA was inhibited in the presence of Trim44. The knockdown of Trim44 exerted the opposite effect. However, K63-linked polyubiquitination displayed no difference either with or without Trim44 expression. Taken together, Trim44 acts as a positive regulator of the RIG-I–mediated signaling pathway, which stabilizes VISA by inhibiting K48-linked polyubiquitination.

The Trim family has been shown to play an important role in innate immunity. Notably, some of the Trim family members regulate antimicrobial responses through ubiquitination. Trim8 positively regulates TNF-α and IL-1β–triggered NF-κB activation by targeting TAK1 for K63-linked polyubiquitination (18). Trim23 mediates K27-linked ubiquitin conjugation to NEMO to regulate TLR3- and RIG-I/MDA5–mediated antiviral innate and inflammatory responses (19). Trim25 induces the K63-linked ubiquitination of RIG-I to elicit the host antiviral innate immunity (21). Trim38 targets NAP1 for K48-linked ubiquitination and subsequent proteasome-mediated degradation (24). Trim56, which interacts with STING, mediates the K63-linked polyubiquitination of STING and is required for dsDNA-mediated innate immune responses (27). In these cases, the Trim family members act as E3 ubiquitin ligases and promote the polyubiquitination of their target proteins. However, the structure of Trim44 is very different from that of these Trim family members. Trim44 does not have the typical RING finger domain. Instead, the N-terminal region of Trim44 contains a ZF UBP domain (14). This particular domain is found in many proteins in the ubiquitin-proteasome system, especially shared by the members of the ubiquitin-specific protease family, which typically remove ubiquitin from substrates before proteasomal recognition, thereby resulting in the inhibition of substrate degradation (32, 33). For example, ubiquitin-specific protease 7 stabilizes p53 by deubiquitinating it and protecting it from proteasomal degradation (34). The ZF UBP domain has been shown to bind to ubiquitin with high affinity (35). Therefore, Trim44 may function as a deubiquitinating enzyme and regulate the deubiquitination and stabilization of VISA. However, the regulation of VISA expression upon viral infection is a complicated process. It has been reported that several molecules are able to promote the ubiquitination and degradation of VISA, such as RNFS (36), RNF125 (37), ICH, PSMA7 (38), and PCBP2 (31). Therefore, another possible mechanism by which Trim44 stabilizes VISA involves the interference of Trim44 with the modification of VISA. Even if Trim44 is a deubiquitinating enzyme, the association between Trim44 and other E3 ligases is an interesting field. Further examination is required to characterize the role of Trim44 in the regulation of VISA stability.

Taken together, our findings indicate that Trim44 is a new positive regulator of the virus-triggered VISA-mediated signaling pathway. Trim44 is induced by viral infection and stabilizes VISA by inhibiting the ubiquitination and degradation of VISA. These data provide new evidence of the important role that the Trim family plays in the antiviral response by regulating the ubiquitination of critical regulatory components.

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Disclosures

The authors have no financial conflicts of interest.

References


