Ubiquitination and Degradation

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The E3 Ubiquitin Ligase RNF5 Targets Virus-Induced Signaling Adaptor for Ubiquitination and Degradation

Bo Zhong, Yu Zhang, Bo Tan, Tian-Tian Liu, Yan-Yi Wang, and Hong-Bing Shu

Viral infection activates transcription factors, such as NF-κB and IFN regulatory factor 3, which collaborate to induce type I IFNs and elicit innate antiviral response. Virus-induced signaling adaptor (VISA) has been identified as a critical adaptor required for virus-triggered induction of type I IFNs. In this study, we showed that the E3 ubiquitin ligase RING-finger protein 5 (RNF5) interacted with VISA at mitochondria in a viral infection-dependent manner. Domain mapping experiments indicated that the C-terminal transmembrane domain of VISA was required for its interaction with RNF5. RNF5 targeted VISA at K362 and K461 for K48-linked ubiquitination and degradation after viral infection, whereas knockdown of RNF5 reversed virus-induced down-regulation of VISA at the early phase. These findings suggest that RNF5-mediated ubiquitination and degradation of VISA is one of the mechanisms of the regulation of virus-triggered induction of type I IFNs and cellular antiviral response. The Journal of Immunology, 2010, 184: 6249–6255.

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Abbreviations used in this paper: AIP4, atrophin-1 interacting protein 4; oM, anti-MITA; CARD, caspase recruitment domain; ER, endoplasmic reticulum; HA, hemagglutinin; IFN, IFN-stimulated gene; MITA, mediator of IFR3 activation; PCBP2, poly(rC) binding protein 2; PSMAT7, proteasome subunit a type-7; RING, ring-finger; RNAi, RNA interference; RNF5, RING-finger protein 5; SeV, Sendai virus; TM, transmembrane; TRAF, TNFR-associated factor; VISA, virus-induced signaling adaptor; VSV, vesicular stomatitis virus.

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Materials and Methods

Reagents and Abs

Mouse mAbs against Flag, hemagglutinin (HA), and β-actin (Sigma-Aldrich, St. Louis, MO) and apoptosis inducing factor and KDEL (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased from the indicated manufacturers. Sendai virus (SeV), vesicular stomatitis virus (VSV), and
rabbit anti-VISA, mouse anti-RNF5, and mouse anti-MITA Abs were previously described (13, 18, 25). Goat anti-Itch was generously provided by Dr. Zhengfan Jiang (Peking University, Beijing, China).

**Constructs**

Mammalian expression plasmids for HA- or Flag-tagged VISA and its mutants, MITA, and RNF5 and its mutants were previously described (13, 18, 25). Mammalian expression plasmid for ankyrin repeat and death domain containing 1A was made in a CMV promoter-based expression plasmid by standard molecular biology techniques. Site-directed mutation of mammalian expression plasmids for human HA- or Flag-tagged VISA were constructed by standard molecular biology techniques. The RNF5 (C42S) was a generous gift from Dr. Douglas Cyr (University of North Carolina, Chapel Hill, NC). HA- or Flag-tagged poly(C) binding protein 2 (PCBP2) and Itch and Itch-RNA interference (RNAi) were provided by Dr. Zhengfan Jiang (Peking University) and previously described (26).

**Cell fractionation assays**

The cell fractionation assay was performed as previously described with few changes (25). The 293 cells (5 \times 10^7) infected with SeV or left uninfected were washed with PBS followed by dousing 40 times in 2 ml homogenization buffer (ApplyGen, Beijing, China). The homogenate was centrifuged at 500 \times g for 10 min. The supernatant (S5) was centrifuged at 5000 \times g for 10 min to precipitate mitochondria (P5K). The supernatant from this step (S5K) was further centrifuged at 50,000 \times g for 30 min to yield P50K, which contains the membrane fraction, and S50K, which mainly consists of cytosol.

**Coimmunoprecipitation and immunoblot analysis**

For transient transfection and coimmunoprecipitation experiments, 293 cells (2 \times 10^6) were transfected for 20 h. The transfected cells were lysed in 1 ml lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF]. For each immunoprecipitation, 0.4 ml lysate was incubated with 0.5 μg indicated Ab or control IgG and 30 μl protein G-Sepharose in 20% ethanol (GE Healthcare, Piscataway, NJ) for 2 h. The Sepharose beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were analyzed by standard immunoblot procedures.

For endogenous coimmunoprecipitation experiments, 293 cells (5 \times 10^7) were infected with SeV for the indicated times or left uninfected. The coimmunoprecipitation and immunoblot experiments were performed as described above.

**Ubiquitination assays**

For in vivo ubiquitination experiments, we adopted a two-step immunoprecipitation strategy (25). First-round immunoprecipitation was performed as described above. The immunoprecipitates were re-extracted in lysis buffer containing 1% SDS and denatured by heating for 5 min. The supernatants were diluted with regular lysis buffer until the concentration of SDS was decreased to 0.1%. The diluted supernatants were re-immunoprecipitated with the indicated Abs, and the immunoprecipitates were analyzed by immunoblots with the anti-ubiquitin Ab.

For in vitro ubiquitination experiments, proteins were expressed with a TNT Quick Coupled Transcription/Translation Systems kit (Promega, Madison, WI) as the manufacturer’s instructions described. Ubiquitination was analyzed with an ubiquitination kit (Enzo Life Sciences, Farmingdale, NY) following the protocols recommended by the manufacturer.

**Results**

**Domain mapping of the association of RNF5 and VISA**

Previously, we found that RNF5 interacts with VISA in a mammalian overexpression system (25). However, the significance of the association between RNF5 and VISA has not been determined. We first mapped the domains of VISA that are required for its interaction with RNF5. As shown in Fig. 1A and 1B, full-length VISA and its deletion mutants containing the C-terminal transmembrane domain could interact with RNF5, whereas the mutants lacking the

![FIGURE 1.](http://www.jimmunol.org/) RNF5 interacts with VISA. A, A schematic presentation of full-length VISA, its mutants, and their abilities to interact with RNF5. The TM domain of VISA is required for its interaction with RNF5. The 293 cells (2 \times 10^6) were transfected with the indicated plasmids (5 μg each). Coimmunoprecipitations were performed with anti-Flag or control IgG. Immunoblot analysis was performed with anti-HA (upper panels). Expression levels of the proteins were analyzed by immunoblot analysis of the lysates with anti-HA and anti-Flag (lower panels). C, A schematic presentation of full-length RNF5, its mutants, and their abilities to interact with VISA. D, Interactions between RNF5 mutants and VISA. Experiments were performed similarly as in B. CARD, caspase recruitment domain; TM, transmembrane.
transmembrane domain lost their ability to associate with RNF5. These data suggest that the C-terminal transmembrane domain of VISA is required for its interaction with RNF5.

In our previous study, we also found that the transmembrane domain of RNF5 is required for RNF5–MITA association (25). We next examined which domains of RNF5 are required for its interaction with VISA. Interestingly, deletion of either the N-terminal ring-finger (RING) domain (aa 1–50) or the C-terminal transmembrane domain (aa 169–180) of RNF5 had no marked effects on its interaction with VISA. In contrast, the truncation mutant RNF5 (51–168), which lacks both the RING and the transmembrane domains, failed to interact with VISA (Fig. 1D). These results suggest that the intermediate domain plus either the RING or the transmembrane domain of RNF5 are sufficient for its interaction with VISA.

We next determined whether VISA interacts with RNF5 in uninfected cells and the effects of viral infection on the interaction. Endogenous coimmunoprecipitation experiments indicated that VISA did not interact with RNF5 without stimulation but was weakly associated with RNF5 at 4 h after viral infection and was strongly associated with RNF5 at 8 h after viral infection (Fig. 2A). Interestingly, their association was diminished at 12 h after viral infection (Fig. 2A). Because VISA has been demonstrated to be exclusively localized to the outer membrane of mitochondria (14), we further determined whether their association occurs at the mitochondria. We isolated the mitochondria and ER fractions and performed coimmunoprecipitation assays. As expected, although RNF5 distributed to both the mitochondria and the ER fractions, RNF5 interacted with VISA at the mitochondria but not the ER fraction (Fig. 2B). These results suggest that VISA is associated with RNF5 at the mitochondria in a viral infection-dependent manner.

**RNF5 targets VISA for ubiquitination and degradation**

Because RNF5 is an E3 ubiquitin ligase and associates with VISA at the mitochondria in a viral infection-dependent manner, we determined whether RNF5 could catalyze ubiquitination of VISA. As shown in Fig. 3A, wild-type RNF5 but not the inactive mutant (C42S) mediated ubiquitination of VISA. To exclude the possibility that the polyubiquitin-associated VISA is due to the association of VISA with MITA or RNF5, we performed two lines of experiments. First, we examined RNF5-mediated ubiquitination of VISA with or without RNAi-mediated knockdown of MITA and found that knockdown of MITA had no marked effect on RNF5-mediated ubiquitination of VISA (Fig. 3B). Second, we examined the reimmunoprecipitation system used in our study. The results indicated that the denaturation and reimmunoprecipitation assay completely disrupted the interaction between VISA and RNF5, but the polyubiquitin modification of VISA remained intact (Fig. 3C). These data suggest that RNF5 mediates ubiquitination of VISA.

Consistent with these observations, RNF5 but not its mutant RNF5(C42S) or MITA caused downregulation of VISA in a dose-dependent manner (Fig. 3D). Furthermore, SeV and VSV infection resulted in ubiquitination and downregulation of endogenous VISA, and these were reversed by RNAi-mediated knockdown of RNF5 (Fig. 3E), suggesting viral infection-caused downregulation of VISA was not viral type-specific. Kinetic experiments of VISA expression indicated that the expression of VISA was gradually downregulated from 6–24 h after viral infection, and knockdown of RNF5 slowed the virus-triggered downregulation of VISA (Fig. 3F). In contrast, MITA was degraded at 6–9 h after SeV infection, and knockdown of RNF5 abolished the effect. Consistently, knockdown of RNF5 maintained the VISA–MITA interaction at 8 h after SeV infection (Fig. 3G). These data suggest that RNF5 targets VISA for ubiquitination and degradation after viral infection.

**RNF5-mediated downregulation of VISA depends on the proteasome**

Because proteins with the K48-linked or the K63-linked polyubiquitin chains could be targeted for degradation in proteasome- and lysosome-dependent pathways, respectively, we next determined whether RNF5 catalyzed K48- or K63-linked ubiquitination of VISA. We previously made the ubiquitin mutant constructs in which all of the lysine residues except Lys48 (ubiquitin-K48) or Lys63 (ubiquitin-K63) were mutated into arginines (25). We transfected these ubiquitin mutants into 293 cells and examined whether they could modify VISA. RNF5 catalyzed VISA ubiquitination with wild-type ubiquitin and ubiquitin-K48 but not with ubiquitin-K63 (Fig. 4A). Consistently, the proteasome inhibitor MG132 could reverse RNF5-mediated downregulation of overexpressed VISA or SeV-induced downregulation of endogenous VISA (Fig. 4B, 4C). These results suggest that RNF5 targets VISA for K48-linked ubiquitination and proteasome-dependent degradation after viral infection.

**FIGURE 2.** Effect of viral infection on endogenous VISA–RNF5 interaction. A, Endogenous interaction between VISA and RNF5. The 293 cells (5 × 10^5) were left uninfected or infected with SeV for the indicated time points. The cells were lysed, and the lysates were immunoprecipitated with anti-RNF5 or control serum. The immunoprecipitates were analyzed by immunoblot with anti-VISA (upper panel). The expression levels of the endogenous proteins were detected by immunoblot analysis with anti-MITA and anti-RNF5 (lower panels). B, RNF5 interacts with VISA at mitochondria after viral infection. The 293 cells (1 × 10^5) were left uninfected or infected with SeV for the indicated time points and fractionated as shown in the diagram (upper schematic presentation). The fractions were lysed, the lysates were immunoprecipitated with anti-RNF5, and the immunoprecipitates were analyzed by immunoblot with anti-VISA (top panel). A fraction of lysate was taken for immunoblot analysis to detect the expression levels of the indicated proteins (lower panels).
RNF5 targets K362 and K461 of VISA for ubiquitination

During our coimmunoprecipitation experiments, we routinely observed that RNF5 but not the C42S inactive mutant caused shifts of VISA(361–540) to higher molecular bands (Figs. 1B, 5A). In addition, RNF5 caused ubiquitination of wild-type VISA but not the mutant VISA(D361–480), suggesting the possibility that the ubiquitination sites of VISA are located in the region of aa 361–480 of VISA (Fig. 5B). Sequence analysis identified four lysine residues in this region as potential ubiquitination sites.
residues in this region, which are K362, K371, K420, and K462. We made VISA mutants in which the lysine residues were mutated to arginines individually or simultaneously and examined their ubiquitination by RNF5. As shown in Fig. 5C, mutation of K362 or K461 to arginine reduced RNF5-mediated ubiquitination of VISA, whereas simultaneous mutation of both K362 and K461 to arginines (K362/461R) abolished RNF5-catalyzed ubiquitination. Consistently, RNF5 caused degradation of wild-type VISA but not VISA(K361/461R) (Fig. 5C). These data suggest that RNF5 targets VISA at K362 and K461 for ubiquitination and degradation.

**RNF5 and Itch independently target VISA for ubiquitination**

Recently, it has been reported that the homologous to E6-AP carboxyl terminus domain containing E3 ubiquitin ligase Itch (also called atrophin-1 interacting protein 4 [AIP4]) catalyzes ubiquitination of VISA and this process is mediated by PCBP2. To
determine whether RNF5 and Itch function independently or together for ubiquitination of VISA, we performed two lines of experiments. First, we performed in vitro ubiquitination assays with recombinant proteins. The results indicated that RNF5 directly catalyzed ubiquitination of VISA and the E2 Ubc5 (UbcH5a, UbcH5b, or UbcH5c) was required for this process (Fig. 6A). Second, RNF5 could ubiquitinate VISA in AIP4 knockdown cells, whereas AIP4 and PCBP2 could also catalyze ubiquitination of VISA in RNF5 knockdown cells (Fig. 6B). These data suggest that RNF5 and Itch independently catalyze ubiquitination of VISA. To examine whether RNF5 and Itch function redundantly for VISA degradation, we examined the effects of knockdown of RNF5 versus Itch on the stability of VISA. As shown in Fig. 6C, SeV-induced downregulation of VISA was inhibited by knockdown of RNF5 at 8 h but less at 16 h after SeV infection, whereas knockdown of Itch inhibited degradation of VISA from 8–16 h after SeV infection. These data suggest that RNF5 and Itch function in a temporal manner to target VISA for ubiquitination after viral infection.

Discussion

Our previous studies have demonstrated that the ubiquitin ligase RNF5 regulates antiviral response by mediating degradation of the adaptor protein MITA (25). In this report, we found that RNF5 was associated with VISA in a viral infection-dependant manner in untransfected cells. Domain mapping experiments suggested that the transmembrane domain of VISA was required for its interaction with RNF5. In contrast to the observation that the transmembrane domain of RNF5 is required for its association with MITA, we found that the intermediate domain plus either the RING or the transmembrane domain of RNF5 was sufficient for interaction with VISA.

RNF5 is an E3 ubiquitin ligase, and Cys^{42} of RNF5 is critical for its ligase activity (27). Consistently, wild-type RNF5 but not the RNF5(C42S) mutant catalyzed ubiquitination and downregulation of VISA. Moreover, RNF5 also mediated virus-triggered ubiquitination and degradation of VISA, as demonstrated by the observation that RNAi-mediated knockdown of RNF5 diminished these effects. Further studies indicated that RNF5 catalyzed K48-linked ubiquitination of VISA, and SeV infection-induced and RNF5-mediated downregulation of VISA was reversed by treatment with the proteasome inhibitor MG132. Site-directed mutagenesis indicated that RNF5 ubiquitinated VISA at K362 and K461. Consistently, VISA(K362/461R) was resistant to RNF5-mediated degradation. These results suggest that RNF5 targets VISA for K48-linked ubiquitination and proteasome-dependent degradation after viral infection.

Our experiments indicated that VISA was degraded gradually from 6–24 h after viral infection. However, knockdown of RNF5 reversed the degradation of VISA at 6–12 h after viral infection. Consistently, a fraction of RNF5 translocated from ER to mitochondria and interacted with VISA at 4–8 h after viral infection, similar to its interaction with MITA (25). Previously, it has been reported that the E3 ubiquitin ligase RNF125 is induced by viral infection or IFN-α stimulation and catalyzes ubiquitination of RIG-I, MDA5, and VISA (22). Recently, two studies also reported that Itch and proteasome subunit α type-7 (PSMA7) mediate ubiquitination and degradation of VISA and their expression is induced by viral infection (26, 28). Therefore, it is possible that viral infection leads to translocation of RNF5 from ER to mitochondria, making it accessible to MITA and VISA and mediating the early negative regulation of virus-triggered induction of type I IFNs by down-regulating VISA and MITA, whereas RNF125, Itch, and PSMA7 are induced by viral infection and target VISA for ubiquitination at the late phase of viral infection. In this context, we observed that knockdown of RNF5 inhibited degradation of VISA at the early phase of viral infection. At the late phase of viral infection, the expression of Itch (together with PCBP2) is induced, which may account for the degradation of VISA. However, it should be noted that knockdown of RNF5 or Itch did not fully restore expression of VISA at 8 or 16 h after SeV infection, probably due to the fact that knockdown efficiency is not 100% or alternatively other molecules, such as PSMA7 or RNF125, existing for ubiquitination and degradation of VISA.
degradation of VISA. Further studies are required to determine the contributions of these molecules in mediating degradation of VISA.

A recent report suggests that viral infection causes both K48- and K63-linked ubiquitination of VISA. However, the E3 ubiquitin ligases responsible for these processes were not identified (29). The K63-linked polyubiquitin chain at K500 of VISA leads to its recruitment of inhibitor of NF-κB kinase ε to the mitochondria and activation of NF-kB and several ISGs (29). In our study, mutation of K500 to arginine had no marked effects on RNF5-mediated ubiquitination of VISA, suggesting that K500 is not a target for K48-linked ubiquitination by RNF5. Our results suggest that RNF5 is an E3 ubiquitin ligase for K48-linked ubiquitination of VISA. Further investigations are required to characterize the E3 ubiquitin ligase(s) responsible for K63-linked ubiquitination of VISA.

It has been demonstrated that VISA plays a key role in virus-triggered induction of type I IFNs (13–16, 30, 31). Therefore, the regulation of VISA is critical for the proper control of antiviral immune response. The identification of RNF5 as an E3 ubiquitin ligase for VISA ubiquitination and degradation after viral infection provides a previously undiscovered strategy for the control of excessive cellular antiviral response.

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Disclosures
The authors have no financial conflicts of interest.

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