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

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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 downregulation 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.

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. 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 × 10^7) infected with SeV or left uninfected were washed with PBS followed by dosing 40 times in 2 ml homogenization buffer (ApplyGen, Beijing, China). The homogenate was centrifuged at 500 × g for 10 min. The supernatant (S5) was centrifuged at 5000 × g for 10 min to precipitate mitochondria (P5K). The supernatant from this step (S5K) was further centrifuged at 50,000 × 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 × 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 × 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/.../by-guest-on-April-2017/...6250.html)
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(C42S), 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 untransfected 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.
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, 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.

![FIGURE 4](image)

**FIGURE 4.** RNF5-mediated downregulation of VISA depends on the proteasome. A, RNF5 catalyzes K48-linked polyubiquitination of VISA. The 293 cells (2 × 10⁶) were transfected with the indicated plasmids. Twenty hours after transfection, immunoprecipitation, reimmunoprecipitation, and immunoblot analysis were performed with the indicated Abs (upper panel). The expression of the proteins was examined by immunoblots with the indicated Abs (lower panels). B, MG132 blocks RNF5-mediated downregulation of endogenous VISA. The 293 cells (1 × 10⁶) were transfected with the indicated plasmids. Twenty hours after transfection, cells were treated with DMSO or MG132 (20 μM) for 6 h before immunoblot analysis was performed. C, MG132 reverses SeV-induced downregulation of VISA. The 293 cells (1 × 10⁶) were left untreated or infected with SeV. Two hours later, cells were treated with DMSO or MG132 (20 μM) for 6 h before immunoblot analysis was performed.

![FIGURE 5](image)

**FIGURE 5.** Mapping of RNF5-targeted ubiquitination sites of VISA. A, Wild-type RNF5 but not its enzyme inactive mutant RNF5(C42S) causes shift of VISA(361–540) to higher molecular bands. The 293 cells (2 × 10⁶) were transfected with the indicated plasmids. Twenty hours later, cells were lysed, and immunoprecipitation and immunoblot analysis were performed with the indicated Abs. B, RNF5 catalyzes ubiquitination of wild-type VISA but not VISA (Δ361–480). The 293 cells (2 × 10⁶) were transfected with the indicated plasmids. Twenty hours after transfection, immunoprecipitation, reimmunoprecipitation, and immunoblot analysis were performed with the indicated Abs (upper panel). The expression of the proteins was examined by immunoblots with the indicated Abs (lower panels). C, Mapping of RNF5-targeted ubiquitination sites of VISA. Experiments were performed as in A.
Our previous studies have demonstrated that the ubiquitin ligase RNF5 regulates antiviral response by mediating ubiquitination of the adaptor protein MITA (25). In this report, we found that RNF5 is 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\textsuperscript{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 the E2 Ubc5 (UbcH5a, UbcH5b, or UbcH5c). VISA and RNF5 were translated in vitro, and the indicated E2s were added for ubiquitination assays. Ubiquitin-conjugated VISA was detected by immunoblot with HRP–streptavidin (upper panel). Before ubiquitination analysis, the levels of the translated proteins were detected with the indicated Abs (lower panels). RNF5 catalyzes ubiquitination of VISA independent of Itch. The 293 cells (2 x 10\textsuperscript{6}) were transfected with the indicated plasmids. Twenty-four hours after transfection, immunoprecipitation, reimmunoprecipitation, and immunoblot analysis were performed with the indicated Abs (upper panel). The expression of the proteins was examined by immunoblots with the indicated Abs (lower panels). C, Effects of RNF5 or Itch knockdown on the stability of VISA. The 293 cells (4 x 10\textsuperscript{6}) were transfected with the indicated plasmids (8 μg each). Twenty-four hours after transfection, cells were infected with SeV for the indicated time points before immunoblot analysis was performed.
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 bκ kinase ε to the mitochondria and activation of NF-κB 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 will be needed 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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