Virus-triggered Ubiquitination of TRAF3/6 by cIAP1/2 Is Essential for Induction of Interferon-β (IFN-β) and Cellular Antiviral Response*

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Viral infection causes activation of transcription factors NF-κB and IRF3, which collaborate to induce type I interferons (IFNs), and cellular antiviral response. Here we show that knockdown of the E3 ubiquitin ligases cIAP1 and cIAP2 markedly inhibited virus-triggered activation of IRF3 and NF-κB as well as IFN-β induction. Knockdown of cIAP1 and cIAP2 also inhibited cytoplasmic dsRNA-triggered cellular antiviral response. Endogenous coimmunoprecipitation experiments indicated that viral infection caused recruitment of cIAP1 and cIAP2 to TRAF3, TRAF6, and VISA. Furthermore, we demonstrated that cIAP1- and cIAP2-mediated virus-triggered ubiquitination of TRAF3 and TRAF6. These findings suggest that virus-triggered ubiquitination of TRAF3 and TRAF6 by cIAP1 and cIAP2 is essential for type I IFN induction and cellular antiviral response.

Viral infection results in induction of type I interferons (IFNs), including IFN-β and IFN-α family cytokines (1–3). Type I IFNs activate the JAK-STAT signal transduction pathways, leading to transcriptional induction of a wide range of downstream antiviral genes and subsequent innate antiviral response (4, 5).

Signaling pathways responsible for virus-triggered IFN induction have been extensively investigated during the past decade. In limited types of cells, viral RNA is detected by certain membrane-bound Toll-like receptors (TLRs). However, in most cell types, viral RNA is recognized by cytoplasmic pattern recognition receptors (PRRs) RIG-I and MDA5. Both RIG-I and MDA5 contain two CARD modules at their N terminus and a DExD/H-box RNA helicase domain at their C terminus. Upon viral infection, the RNA helicase domains of RIG-I and MDA5 serve as intracellular viral RNA receptors. The recognition of viral RNA by RIG-I and MDA5 leads to their recruitment to the downstream mitochondrion-located CARD-containing adapter protein VISA (also known as MAVS, IPS-1, and Cardif) (6–10). VISA is constitutively associated with another mitochondrion-associated adapter protein MITA/STING (11, 12).

Various studies have demonstrated that VISA plays a central role in assembling a complex that activates distinct signaling pathways leading to NF-κB and IRF3 activation, respectively. VISA is associated with TRAF2 and TRAF6 through its TRAF-interaction motifs. It has been shown that TRAF2 and TRAF6 facilitate Lys-63-linked polyubiquitination of RIP and NEMO/IKKγ, respectively, and these processes cause activation of IKK and subsequent NF-κB (13–15). VISA is also associated with TRAF3, another member of the TRAF protein family (16). Gene knock-out studies have demonstrated that TRAF3 is essential in virus-triggered IRF3 activation and type I IFN induction (17, 18). However, how TRAF3 is regulated in virus-triggered signaling pathways is still enigmatic.

Several studies have suggested that ubiquitination is a central rhythm of regulation of the virus-triggered IFN induction pathways. It has been shown that the E3 ubiquitin ligase TRIM25 catalyzes Lys-63-linked ubiquitination of RIG-I, and this ubiquitination is essential for the interaction of RIG-I with VISA as well as for its ability to signal (19). The Riplet/REUL E3 ubiquitin ligase also targets RIG-I for ubiquitination, which positively regulates RIG-I-mediated signaling (20, 21). In contrast, the E3 ubiquitin ligase RNF125 catalyzes Lys-48-linked ubiquitination of RIG-I and negatively regulates RIG-I-mediated signaling (22). MITA and IRF3 are ubiquitinated by RNF5 and RBCK1, respectively, and subsequently degraded by proteasome-dependent processes (23, 24). The E3 ubiquitin ligase Nrdp1 catalyzes the ubiquitination of TBK1, leading to its activation (25).

cIAP1 and cIAP2 are E3 ubiquitin ligases that were firstly identified as signaling components associated with TRAF1 and TRAF2 and recruited to the TNF receptors TNFR1 and TNFR2 upon ligand stimulation (26, 27). Recently, it has been demonstrated that in unstimulated cells, TRAF2/3 and cIAP1/2 form a cytoplasmic complex, which constitutively ubiquitinates NIK and promotes its proteasome-dependent degradation (28, 29). Upon stimulation by TNF family members, such as BAFF and CD40L, the TRAF2/3-cIAP1/2 complex is recruited to the receptors, where cIAP1/2 mediate Lys-48-linked ubiquitination and degradation of TRAF3. Such ubiquitination of TRAF3 is critical for activation of downstream MEKK and MAPK kinase cascades (30). In this report, we found that the E3 ubiquitin ligases cIAP1 and cIAP2 caused TRAF3 ubiquitination following viral infection and this is essential for virus-triggered IRF3 activation as well as IFN-β induction. These findings provide insights into the mechanisms on how TRAF3 is positively

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1. The abbreviations used are: IFN, interferon; HA, hemagglutinin; PBS, phosphate-buffered saline; SM, Smac mimetic; MOI, multiplicity of infection; RNAi, RNA interference; ISRE, interferon-stimulated response element; VSV, vesicular stomatitis virus; VISA, virus-induced signaling adapter.
regulated in the virus-triggered signaling pathways and cellular antiviral response.

**EXPERIMENTAL PROCEDURES**

*Reagents*—Antibodies against Myc tag (Cell Signaling); cIAP1, cIAP2, and cIAP Pan (R&D); TRAF3, AIF, and ubiquitin (Santa Cruz Biotechnology); Flag, HA, β-actin, and α-tubulin (Sigma); horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG (Thermo); and HRP-conjugated anti-goat IgG (ZhongShanJinQiao) were purchased from the indicated companies. Mouse antisera against cIAP1, cIAP2, TRAF3 were raised against the respective recombinant human proteins. SeV, VSV, and rabbit anti-VISA antibody were previously described (10, 12, 34–36). SM was kindly provided by Xiaodong Wang (University of Texas Southwestern Medical School). Poly(I:C) (Invitrogen), B-DNA (Amersham Biosciences), and NEM (Sigma) were purchased from the indicated manufacturers.

**Constructs**—NF-κB, ISRE, IRF1, and the IFN-β promoter luciferase reporter plasmids, mammalian expression plasmids for RIG-I-N, MDA5-N, VISA, TBK1, TRAF3, TRAF6, IRF3, IRF3–5D, P65 were previously described (10, 12, 25, 35, 36). CMV promoter-based mammalian expression plasmids for Myc- or Flag-tagged cIAP1 and cIAP2 were constructed by standard molecular biology techniques. Mammalian expression plasmids for Myc-tagged cIAP1(H588A) and cIAP2(H574A) or Flag-tagged TRAF6(C70/73S) were constructed by standard site-directed mutagenesis method. Mammalian expression plasmids for HA-tagged Lys-48- and Lys-63-ubiquitin mutants (all lysine residues except Lys-48 or Lys-63 are mutated) were made by site-directed mutagenesis (24).

**Transfection and Reporter Assays**—The 293 cells (1 × 10^6) were seeded on 12-well plates and transfected 16 h later by standard calcium phosphate precipitation. In these experiments, empty control plasmid was added to ensure that the same amount of total DNA was transfected to each well. To normalize for transfection efficiency, 0.1 μl of the indicated antibody and 25 μl of a 1:1 slurry of Gamma Bind G Plus-Sepharose (Amersham Biosciences) for 4 h. The Sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were analyzed by standard immunoblots. For endogenous immunoprecipitation experiments, cells were transfected with poly(I:C) or infected with SeV for the indicated times or left untreated. Cells were lysed in 5 ml of lysis buffer, and the lysate was incubated with 1 μl of the indicated antisera or preimmune control serum. The subsequent procedures were carried out as described above.

**Generation and Transfection of Human Primary DCs**—Peripheral blood monocytes (PBMCs) were isolated from healthy human peripheral blood by density gradient separation with the Ficoll-Paque method. The isolated PBMCs were incubated with human CD14-specific antibody conjugated to paramagnetic microbeads (MiltenyiBiotec), and the CD14⁺ monocytes were isolated on the LS columns (Miltenyi Biotech). The monocytes were suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, and 1 μM sodium pyruvate (Invitrogen). For generation of dendritic cells (DCs), the CD14⁺ monocytes were treated with recombiant human (rh) GM-CSF (50 ng/ml) and rhIL-4 (100 ng/ml) (PeproTech) for 6 days. The medium with the cytokines were changed every other day during the differentiation period. Differentiated DCs were harvested and transfected with the human dendritic cell nucleofactor kit (Amaxa). Immediately after transfection, cells were resuspended in the cytokine-containing medium. Twenty-four hours after transfection, cells were infected with SeV for 16 h before reporter assays were performed.

**VSV Plaque Assay**—The 293 cells (1 × 10⁶) were transfected with the indicated plasmids for 36 h prior to VSV infection (MOI of 0.1). At 1 h after infection, cells were washed with PBS for three times and then medium was added. The supernatants were harvested at 24 h after washing. The supernatants were diluted 1:10⁶ and then used to infect confluent BHK21 cells cultured on 24-well plates. At 1-h postinfection, the supernant was removed, and 3% methylcellulose was overlayed. At 3-days postinfection, overlay was removed, cells were fixed with 4% formaldehyde for 20 min, and stained with 0.2% crystal violet. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titer as PFU/ml.

**RNAi**—Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine, Seattle, WA). The target sequences for human cIAP1 cDNA are: 1: GTGATATCTTCTATTC; 2: ATGAAATACACCTGTGTTA; 3: GTACTGAAGAGCAGATA; 4: GAGGAGGAAAGGACCA-GATT. The target sequences for human cIAP2 cDNA are: 1: GAGGAGGAAAGGACCA; 2: GAAAATGGGCTTATTAG-TAGA; 3: GAGACTACAAGAAGAAAGA.

**Subcellular Fractionation**—The cell fractionation experiments were performed as previously described (12). In brief, 293 cells (6 × 10⁶) infected with SeV or left uninfected for buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride). For each immunoprecipitation, 0.9-ml aliquot of lysate was incubated with 0.5 μg of the indicated antibody and 25 μl of a 1:1 slurry of Gamma Bind G Plus-Sepharose (Amersham Biosciences) for 4 h. The Sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were analyzed by standard immunoblots.
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RESULTS

cIAP1/2 Are Required for Virus-triggered IFN-β Induction and Cellular Antiviral Response—Because of the close partnership between cIAP1/2 and TRAF3, a critical mediator of the virus-triggered signaling pathways, we investigated the possible involvement of cIAP1 and cIAP2 in these processes. We constructed three RNAi plasmids for cIAP1 and cIAP2, respectively. These RNAi plasmids could inhibit the expression of transfected and endogenous cIAP1 or cIAP2 to varied levels in 293 cells (Fig. 1A). As shown in Fig. 1B, knockdown of cIAP1 or cIAP2 markedly inhibited SeV-induced activation of the IFN-β promoter in reporter assays. The degree of inhibition was correlated with the efficiency of knockdown of cIAP1 or cIAP2 expression by each RNAi plasmid (The #1 cIAP1-RNAi and #1 cIAP2-RNAi plasmids were used for all the following experiments if the plasmid number was not specifically mentioned. Similar results were obtained with the #3 cIAP1-RNAi and #3 cIAP2-RNAi plasmids) (Fig. 1B). Knockdown of either cIAP1 or cIAP2 also inhibited SeV-triggered transcription of endogenous IFNB1, RANTES, ISG56, and ISG15 genes, and simultaneously knockdown of both cIAP1 and cIAP2 had an accumulative effect (Fig. 1C). Similarly, knockdown of cIAP1 and cIAP2 also inhibited SeV-induced activation of the IFN-β promoter in human primary dendritic cells (DCs) (Fig. 1D). Interestingly, knockdown of cIAP1 and cIAP2 also dramatically inhibited cytoplasmic poly(I:C)- and B-DNA-induced activation of the IFN-β promoter in reporter assays (Fig. 1E). In similar experiments, knockdown of cIAP1 and cIAP2 had no inhibitory effects on IFN-γ-induced IRF1 activation (Fig. 1F). These results suggest that cIAP1 and cIAP2 are specifically required for virus-, cytoplasmic poly(I:C)- and B-DNA-induced IFN-β induction in various cells.

Certain proteins in the virus-triggered IFN induction pathways can induce type I IFNs when overexpressed in mammalian cells. However, overexpression of cIAP1 or cIAP2 did not activate the IFN-β promoter in reporter assays. This property is similar to TRAF3, which is required for virus-triggered induction of type I IFNs but not capable of activating the IFN-β promoter in reporter assays (data not shown).

Because cIAP1 and cIAP2 are critically involved in virus-triggered IFN-β induction, we determined whether these proteins play roles in cellular antiviral response. In plaque assays, knockdown of cIAP1 and cIAP2 enhanced VSV replication and markedly reversed cytoplasmic poly(I:C)-mediated inhibition of VSV replication (Fig. 1G). The combined knockdown of both proteins had an accumulative effect (Fig. 1G). These data sug-
gest that cIAP1 and cIAP2 are required for efficient cellular antiviral response.

Induction of type I IFNs requires coordinated and cooperative activation of the transcription factors IRF3 and NF-κB. Consistently, knockdown of cIAP1 and cIAP2 inhibited SeV-triggered activation of ISRE and NF-κB (Fig. 2, A and B), suggesting that cIAP1 and cIAP2 are required for both virus-triggered IRF3 and NF-κB activation pathways.

**cIAP1/2 Act Downstream of VISA**—The RNA knockdown experiments suggest that cIAP1 and cIAP2 are required for virus-triggered IRF3 and NF-κB activation. We next determined the molecular step at which cIAP1 and cIAP2 are involved. In reporter assays, knockdown of cIAP1 and cIAP2 inhibited activation of the IFN-β promoter induced by overexpression of upstream components RIG-I-N, MDA5-N, and VISA, but not by downstream components TBK1 and IRF3–5D (Fig. 3A). Consistently, knockdown of cIAP1 and cIAP2 inhibited VISA but not TBK1-induced ISRE activation (Fig. 3B), as well as VISA- and TRAF6- but not p65-induced NF-κB activation (Fig. 3C). These data suggest that cIAP1 and cIAP2 act downstream of VISA and upstream of the TBK1 kinase.

**cIAP1/2 Are Associated with TRAF3/6 Following Viral Infection**—Previous studies have demonstrated that TRAF3/6 act downstream of VISA and is required for virus-triggered IFN induction (16). Other studies have suggested that TRAF3/cIAP1/2 form complexes in the cytoplasm (28, 29). Because VISA is a mitochondrial adaptor protein which recruits various components for assembly of a signaling complex after viral infection, we determined whether cIAP1 and cIAP2 were recruited to VISA-associated complex on the mitochondrial membrane. We infected 293 cells with SeV for various time points or left the cells uninfected. Mitochondria from these cells were isolated for coimmunoprecipitation and immunoblotting analysis. The results indicated that cIAP1 and cIAP2 were recruited to VISA at the mitochondria in a viral infection-dependent manner (Fig. 4C). We further determined whether virus-induced association of cIAP1 and cIAP2 with TRAF3 occurs in the cytosol or at the mitochondria. To investigate this, we isolated the cytosolic and mitochondrial fractions and performed coimmunoprecipitation analysis. As shown in Fig. 4D, in the cytosolic fraction, the interaction of cIAP1 and TRAF3 was unchanged before and after viral infection, whereas the association of cIAP1 and TRAF3 at the mitochondria was viral infection-dependent. Interestingly, the level of TRAF3 at the mitochondria was unchanged before and after viral infection (Fig. 4D), suggesting that cIAP1 and TRAF3 are not recruited to the mitochondria as a preformed complex, instead, viral infec-
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FIGURE 4. cIAP1 and cIAP2 associate with TRAF3 and TRAF6 following viral infection. A, effects of SeV infection on endogenous cIAP1/2-TRAF3 and cIAP1/2-TRAF6 interactions. 293 cells (6 × 10⁶) were left uninfected or infected with SeV for the indicated time points. The cells were lysed, and the lysates were immunoprecipitated with anti-TRAF3, anti-TRAF6 or preimmune serum (Pre) as indicated. The immunoprecipitates were analyzed by immunoblots with anti-cIAP1 and anti-cIAP2 (upper panels). The expression levels of the endogenous proteins were detected by immunoblots with the indicated antibodies (lower panels). B, effects of poly(I:C) transfection on endogenous cIAP1/2-TRAF3 and cIAP1/2-TRAF6 interactions. 293 cells (6 × 10⁶) were transfected with poly(I:C) or mock-transfected for 16 h. Co-immunoprecipitation and immunoblot analysis were similarly performed as in A. C, virus-induced recruitment of cIAP1 and cIAP2 to the VISA-associated complex at the mitochondria. 293 cells (6 × 10⁶) were infected with SeV for the indicated times or left uninfected. The mitochondria were isolated by cell fractionation. The mitochondrial lysates were immunoprecipitated. The immunoprecipitates and the lysates were analyzed by immunoblots with the indicated antibodies. D, effects of SeV infection on subcellular location-specific interactions of cIAP1/2 and TRAF3. 293 cells (6 × 10⁶) were infected with SeV for the indicated time points and fractionated into cytosol and mitochondria. Immunoprecipitation and immunoblotting analysis of the fractions were similarly performed as in A.

DISCUSSION

Virus-triggered induction of type I IFNs is crucial for the early innate antiviral response as well as late stage adaptive immunity. This process is delicately regulated in a spatio-temporal manner by various molecules and distinct mechanisms. Ubiquitination has emerged as critical post-translational regulatory mechanisms for activation or attenuation of the virus-triggered IFN response pathways. Previous studies have demonstrated a critical role for the adapter protein TRAF3 and TRAF6 in the virus-triggered induction of type I IFNs (17, 18). However, how TRAF3 and TRAF6 are regulated in virus-triggered IFN induction pathways is not understood. In this report, we demonstrate that TRAF3 and TRAF6 are ubiquitinated by cIAP1 and cIAP2 after viral infection, which are important for virus-triggered IFN induction.

Because of the close partnership between cIAP1/2 and TRAF3/6, critical mediators of the virus-triggered signaling pathways, we investigated the involvement of cIAP1/2 in these processes. Knockdown of either cIAP1 or cIAP2 strongly inhibi-
edited virus-triggered activation of IRF3 and NF-κB, as well as IFN-β induction. Interestingly, knockdown of cIAP1 and cIAP2 also potently inhibited cytoplasmic poly(I:C) and B-DNA-mediated activation of the IFN-β promoter. In plaque assays, knockdown of either cIAP1 or cIAP2 enhanced VSV replication and abrogated cytoplasmic poly(I:C)-mediated cellular antiviral activity. Taken together, these studies suggest that cIAP1/2 play important roles in virus-triggered induction of type I IFNs and cellular antiviral response.

Several lines of evidences suggest that cIAP1/2 target TRAF3/6 in the virus-triggered signaling pathways. Firstly, reporter assays indicated that knockdown of cIAP1/2 inhibited upstream signaling components RIG-I-, MDA5-, VISA- but not downstream TBK1-, IRF3-, and p65-mediated signaling. This is consistent with the molecular orders of TRAF3/6 in the pathways; Second, coimmunoprecipitation experiments indicated that the associations of cIAP1/2 and TRAF3/6 was increased following viral infection. This increase reflected the recruitment of cIAP1/2 to mitochondrial associated TRAF3; Third, several experiments indicated that virus-triggered TRAF3 ubiq-
ubiquitination was abrogated by both RNAi- and Smac mimetic-mediated knockdown of cIAP1/2.

In BAFF and CD40L-mediated signaling pathways, cIAP1/2 catalyze Lys-48-linked ubiquitination of TRAF3 and subsequent degradation of TRAF3 by the proteasomes is critical for activating downstream kinase cascades (30). This mechanism is obviously not the case in virus-triggered signaling pathways. In our experiments, we found that cIAP1/2 could catalyze both Lys-48- and Lys-63-linked ubiquitination of TRAF3/6. Viral infection also induced both Lys-48- and Lys-63-linked ubiquitination of TRAF3, which was completely inhibited by knockdown of cIAP1 and cIAP2. In addition, viral infection or cIAP1/2 overexpression did not cause noticeable degradation of TRAF3/6. These results suggest that degradation of TRAF3/6 is not required for virus-triggered IFN induction pathways. It is possible that polyubiquitins conjugated to TRAF3/6 are capable of activating downstream kinases, such as TBK1, IKKe, and TAK1. Interestingly, time course experiments indicated that virus-triggered ubiquitination of TRAF3/6 was temporally regulated by distinctly linked polyubiquitin moieties: the early stage of ubiquitination was primarily K48-linked whereas the later stage K63-linked. The functional significance of this temporal regulation of ubiquitination is currently unknown.

A recent study suggest that cIAP1/2 ubiquitinate RIP2 and are required for innate immune responses mediated by the PRRs NOD1 and NOD2, which sense intracellular bacteria infection (33). In light of this and our study, it is possible that cIAP1 and cIAP2 are generally involved in innate immune responses mediated by a variety of PRRs.

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