3-26-2010

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Publication Info

Published in *Journal of Biological Chemistry*, Volume 285, Issue 13, 2010, pages 9470-9476.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 13, pp. 9470–9476, March 26, 2010

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

Received for publication, September 29, 2009, and in revised form, January 21, 2010 Published, JBC Papers in Press, January 22, 2010, DOI 10.1074/jbc.M109.071043

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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),[2] 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

* This work was supported by grants from the Chinese 973 Program (2006CB504301, 2010CB911802), the National Natural Science Foundation of China (30630019 and 30921001), the Chinese Science and Technology Key Project (2008ZX10002-014), and the Chinese 111 project (B06018).

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[2] 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-only 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 (~1×10^5) 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 μg of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Approximately 18 h after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (Promega). Firefly luciferase activities were normalized based on *Renilla* luciferase activities. All reporter assays were repeated for at least three times.

RT-PCR—Total RNA was isolated from 293 cells using TRIzol reagent (Invitrogen) and subjected to RT-PCR analysis to measure expression of *IFNB1*, *Rantes*, *ISG56*, *ISG15*, and *GAPDH* genes. Gene-specific primer sequences were as follows: *IFNB1*, 5′-cagcaattttcagtgtcagaagct-3′ and 5′-cagtgactgtactccttggcctt-3′; *Rantes*, 5′-atgaaggtctccgcggcacgcct-3′ and 5′-ctagctcatctccaaagagttg-3′; *ISG15*, 5′-atgggctgggacctgaccgg-3′ and 5′-ttagctccgcccgccaggct-3′; *ISG56*, 5′-acggctgcctaatttacagc-3′ and 5′-agtggctgatatctgggtgc-3′; *GAPDH*, 5′-aaaatcaagtggggcgatgct-3′; and 5′-gggcagagatgatgacccttt-3′.

Immunoprecipitation and Immunoblot Analysis—For transient transfection and coimmunoprecipitation experiments, 293 cells (1×10^6) were transfected with tested expression plasmids for 24 h. The transfected cells were lysed in 1 ml of lysis 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. 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 antiserum 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 mM sodium pyruvate (Invitrogen). For generation of dendritic cells (DCs), the $CD14^+$ monocytes were treated with recombinant 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 (1×10^5) 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 $1{:}10^6$ and then used to infect confluent BHK21 cells cultured on 24-well plates. At 1-h postinfection, the supernatant 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 violetin. 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: GTAGATATCCTCATCTTC; 2: ATGAATACACCTGTGGTTA; 3: GTACTGAAGAAGCCAGATT. The target sequences for human cIAP2 cDNA are: 1: GAGGAGAGAGAAAGAGCAA; 2: GAAATGGGCTTTAGTAGAA; 3: GAGACTACAAGAAGAAAGA.

Subcellular Fractionation—The cell fractionation experiments were performed as previously described (12). In brief, 293 cells (6×10^7) infected with SeV or left uninfected for

various time points were washed with PBS and lysed by dounce-ing for 40 times in 5 ml of homogenization buffer (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 10 mM KCl, 250 mM sucrose). The homogenate was twice centrifuged at 500 × *g* for 10 min. The supernatant was centrifuged at 5,000 × *g* for 10 min to precipitate mitochondria. The supernatant was further centrifuged at 50,000 × *g* for 60 min to generate cytosol. The mitochondria fraction was lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40, protease inhibitor mixture) for 20 min followed by immunoprecipitation or immunoblotting analysis.

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. 1*A*). As shown in Fig. 1*B*, 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. 1*B*). 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. 1*C*). Similarly, knockdown of cIAP1 and cIAP2 also inhibited SeV-induced activation of the IFN-β promoter in human primary dendritic cells (DCs) (Fig. 1*D*). 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. 1*E*). In similar experiments, knockdown of cIAP1 and cIAP2 had no inhibitory effects on IFN-γ-induced IRF1 activation (Fig. 1*F*). 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. 1*G*). The combined knockdown of both proteins had an accumulative effect (Fig. 1*G*). These data sug-

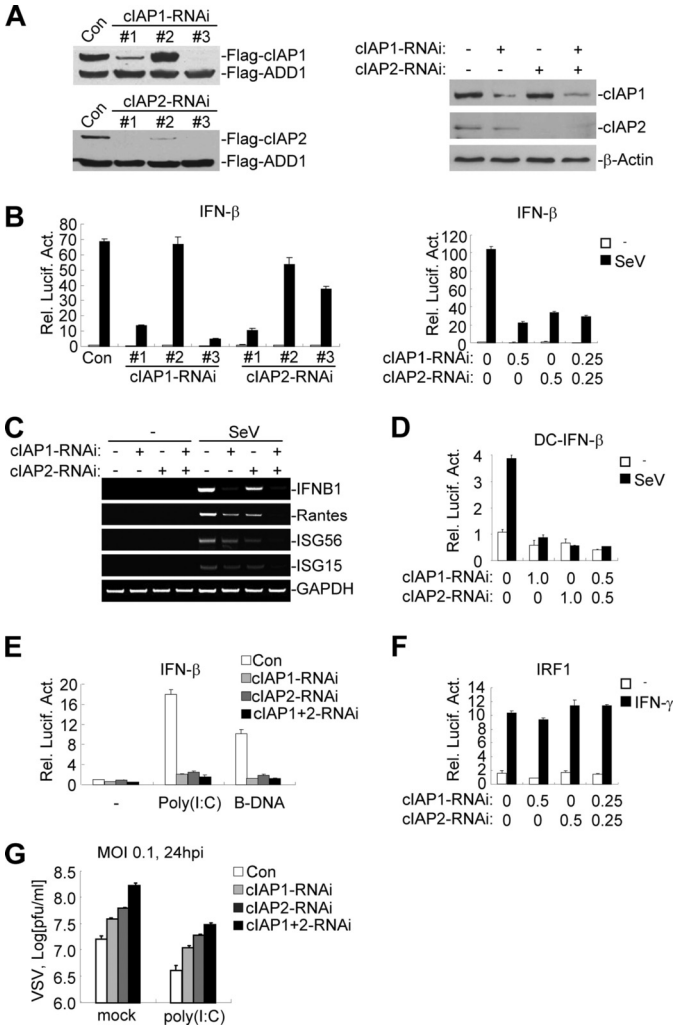

FIGURE 1. **cIAP1 and cIAP2 are required for virus-induced signaling.** *A*, effects of cIAP1 and cIAP2 RNAi plasmids on the expression of cIAP1 and cIAP2. In the *left panels*, 293 cells (2×10^5) were transfected with the indicated expression (0.5 μg each) and RNAi (1 μg each) plasmids for 24 h before immunoblot analysis was performed with anti-Flag. In the right panels, 293 cells (2×10^5) were transfected with the indicated RNAi plasmids (1 μg each) for 24 h. Cell lysates were analyzed by immunoblots with the indicated antibodies. *B*, effects of cIAP1 and cIAP2 RNAi on SeV-induced activation of the IFN-β promoter. 293 cells (1×10^5) were transfected with the indicated RNAi plasmids. Twenty-four hours after transfection, cells were left uninfected or infected with SeV for 10 h before luciferase assays were performed. *C*, effects of cIAP1 and cIAP2 RNAi on SeV-induced expression of downstream genes. 293 cells (2×10^5) were transfected with the indicated RNAi plasmids (1 μg each). Twenty-four hours after transfection, cells were left uninfected or infected with SeV for 10 h before RT-PCR experiments with the indicated primers were performed. *D*, effects of cIAP1 and cIAP2 RNAi on SeV-induced activation of the IFN-β promoter in human primary DCs. The experiments were similarly performed as in *B* except the cells were transfected with the Nucleofactor method. *E*, effects of cIAP1 and cIAP2 RNAi on cytoplasmic dsRNA- and B-DNA-induced activation of the IFN-β promoter. 293 cells (1×10^5) were transfected with the indicated RNAi plasmids. Twenty-four hours after transfection, cells were further transfected with poly(I:C) (1 μg) or B-DNA (1 μg) or mock-transfected for 12 h before luciferase assays were performed. *F*, effects of cIAP1 and cIAP2 RNAi on IFN-γ-induced IRF1 activation. Reporter assays were performed similarly as in *B* except that IRF1 reporter and IFN-γ (100 ng/ml) were used. *G*, knockdown of cIAP1 and cIAP2 increases VSV replication. 293 cells (1×10^5) were transfected with the indicated RNAi plasmid (0.8 μg each). 36 h later, cells were further transfected with poly(I:C) (1 μg) or left untreated. 24 h after poly(I:C) transfection, cells were infected with VSV (multiplicity of infection (MOI), 0.1), and the supernatants were harvested at 24 h post-infection. Supernatants were analyzed for VSV titers with standard plaque assays.

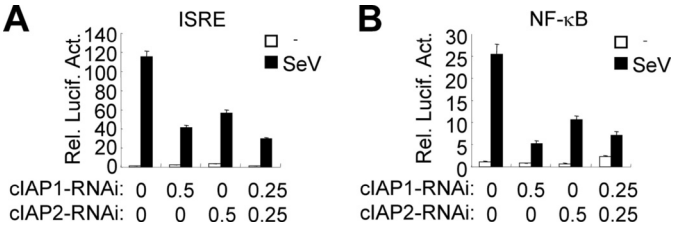

FIGURE 2. **cIAP1 and cIAP2 are required for virus-induced activation of ISRE and NF-κB.** 293 cells (1×10^5) were transfected with ISRE (*A*) or NF-κB (*B*) reporter plasmid, as well as the indicated RNAi plasmids. 24 h after transfection, cells were left uninfected or infected with SeV for 10 h before luciferase assays were performed.

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. 3*A*). Consistently, knockdown of cIAP1 and cIAP2 inhibited VISA- but not TBK1-induced ISRE activation (Fig. 3*B*), as well as VISA- and TRAF6- but not p65-induced NF-κB activation (Fig. 3*C*). 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 our experiments indicated that cIAP1 and cIAP2 function downstream of VISA in virus-triggered signaling, we determined the relationship between TRAF3/6 and cIAP1/2 in the virus-triggered signaling pathways. Endogenous coimmunoprecipitation experiments indicated that cIAP1 constitutively interacted with TRAF3 and TRAF6 and the interactions were markedly enhanced after viral infection (Fig. 4*A*). The interactions between cIAP2 and TRAF3 or TRAF6 were undetectable without stimulation but was easily detected after viral infection (Fig. 4*A*). Interestingly, while the levels of cellular TRAF3, TRAF6 and cIAP1 were not noticeably changed before and after viral infection, the level of cellular cIAP2 was induced after viral infection, which might account for the increased associations between cIAP2 and TRAF3 or TRAF6 after viral infection (Fig. 4*A*). Coimmunoprecipitation experiments indicated that transfection of poly(I:C) to the cytoplasm also induced the interactions between TRAF3/6 and cIAP1/2 (Fig. 4*B*), confirming that activation of the VISA-mediated pathways causes associations of TRAF3/6 and cIAP1/2.

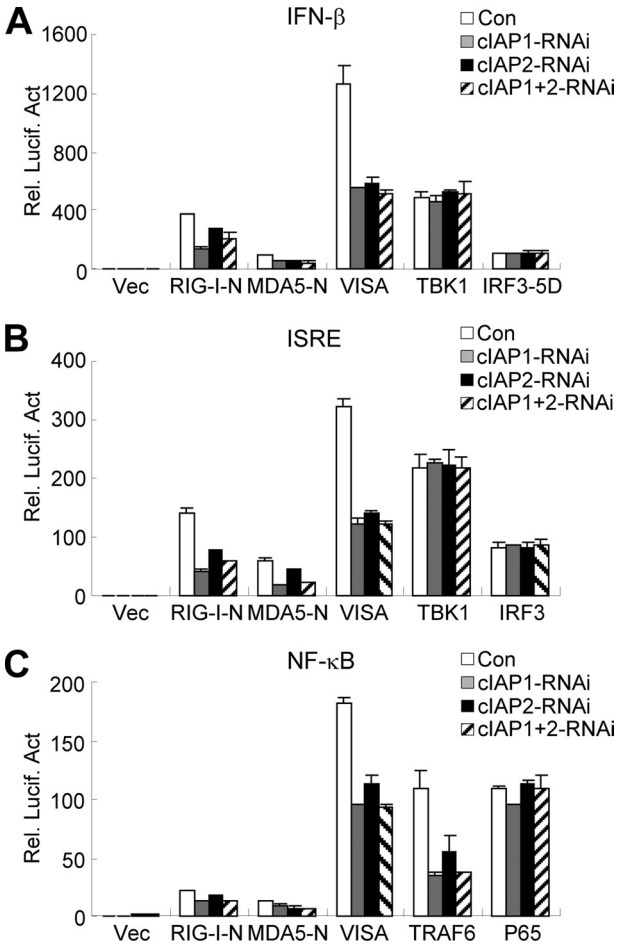

FIGURE 3. **cIAP1 and cIAP2 regulate virus-induced signaling downstream of VISA.** The effects of cIAP1 and cIAP2 RNAi on activation of the IFN-β promoter (*A*), ISRE (*B*), and NF-κB (*C*) mediated by components of the virus-triggered signaling pathways were examined by reporter assays. 293 cells (1×10^5) were transfected with the indicated RNAi plasmids (0.8 μg each). 12 h later cells were selected with puromycin (1 μg/ml) for 24 h and further transfected with the indicated expression and reporter plasmids (0.1 μg each) for 20 h before reporter assays were performed.

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 are 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 coimmunprecipitation and immunoblotting analysis. The results indicated that cIAP1 and cIAP2 were recruited to VISA at the mitochondria in a viral infection-dependent manner (Fig. 4*C*). 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. 4*D*, 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. 4*D*), suggesting that cIAP1 and TRAF3 are not recruited to the mitochondria as a preformed complex, instead, viral infec-

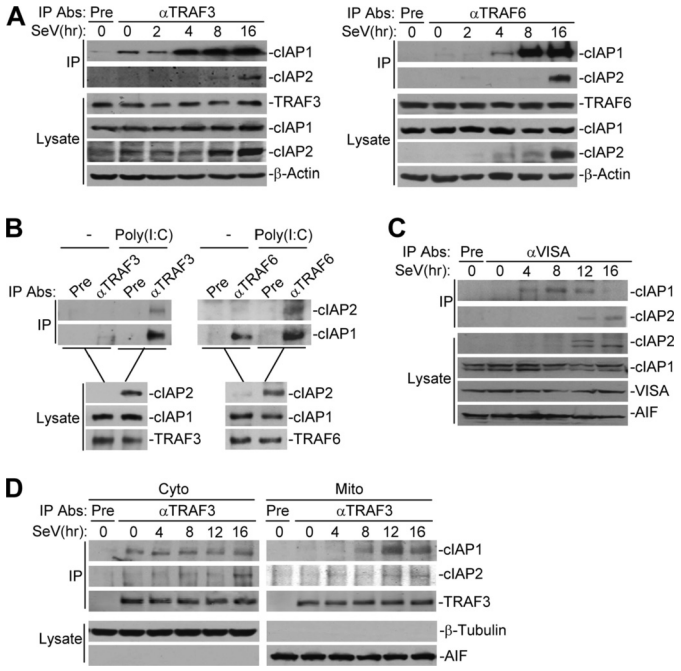

FIGURE 4. **cIAP1 and cIAP2 associate with TRAF3 and TRAF6 following viral infection.** *A*, effects of SeV infection on endogenous cIAP1/2-TRAF3 and cIAP1/2-TRAF6 interactions. 293 cells (6×10^7) were left uninfected or infected with SeV for the indicated time points. The cells were lysed, and the lysates 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 (6×10^7) 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 (6×10^7) 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 (6×10^7) were infected with SeV for the indicated time points and fractionated into cytosol and mitochondria. Immunoprecipitation and immunoblotting analysis with the fractions were similarly performed as in *A*.

tion causes recruitment of cIAP1 to the TRAF3, which already locates at the mitochondria.

cIAP1/2 Mediate Virus-triggered TRAF3 Ubiquitination—It has been shown that certain stimulation, such as BAFF and CD40L, causes cIAP1 and cIAP2 to ubiquitinate TRAF3 (30). Because viral infection caused association of cIAP1/2 with TRAF3 and TRAF6, we determined whether cIAP1 and 2 could ubiquitinate TRAF3/6 following viral infection. Immunoprecipitation experiments indicated that SeV infection caused increased ubiquitination of TRAF3/6 (Fig. 5*A*). Cell fractionation experiments indicated that SeV induced ubiquitination of TRAF3 located at the mitochondria but not in the cytosol (Fig. 5*B*). Using linkage-specific ubiquitin, we further found that SeV induced both Lys-48- and Lys-63-linked ubiquitination of endogenous TRAF3 and TRAF6 (Fig. 5*A*). Interestingly, the dynamics of virus-triggered Lys-48- and Lys-63-linked ubiquitination of TRAF3 and TRAF6 was different. It was obvious that the K48-linked ubiquitination occurred at early stage (6–9 h after viral infection), whereas the Lys-63-linked ubiquitination occurred at a later stage (>9 h after viral infection). These results suggest that Lys-48- and Lys-63-linked ubiquitination of TRAF3 and TRAF6 induced by viral infection is temporally regulated.

We next determined whether cIAP1 and cIAP2 could cause Lys-48- and Lys-63-linked ubiquitination. As shown in Fig. 5*C*, both cIAP1 and cIAP2 mediated Lys-48- and Lys-63-linked ubiquitination of TRAF3, whereas E3 ligase inactive mutants of cIAP1 and cIAP2, cIAP1 (H588A) or cIAP2 (H574A), did not cause TRAF3 ubiquitination. It was noticeable that cIAP1 and cIAP2 were more efficient in mediating Lys-63- than Lys-48-linked ubiquitination (Fig. 5*C*). In these experiments, cIAP1/2 did not ubiquitinate RIG-I, VISA, and IRF3, components in the virus-triggered signaling pathway (data not shown).

Overexpressed TRAF6 was normally highly ubiquitinated by its intrinsic E3 ubiquitin ligase activity (Fig. 5*C*). To determine whether cIAP1 and cIAP2 could cause Lys-48- and Lys-63-linked ubiquitination of TRAF6, we firstly made an E3 ligase-inactive mutant of TRAF6, TRAF6(C70/73S), in which two conserved cysteines are mutated to serines. Using TRAF6(C70/73S) as a substrate, we found that cIAP1 and cIAP2 could also mediate Lys-48- and Lys-63-mediated ubiquitination of TRAF6 (Fig. 5*C*).

We then asked whether endogenous cIAP1 and cIAP2 are required for SeV-induced TRAF3 ubiquitination. As shown in Fig. 6*A*, knockdown of either cIAP1 or cIAP2 by RNAi reduced SeV-induced TRAF3 ubiquitination, whereas knockdown of both cIAP proteins completely abolished SeV-induced TRAF3 ubiquitination, suggesting a redundant function for these two proteins in mediating ubiquitination of TRAF3 after viral infection. To further confirm the role of cIAP1 and cIAP2 in SeV-induced TRAF3 ubiquitination, we used a small molecule mimetic of the pro-apoptotic protein Smac (SM), which binds to cIAP1 and cIAP2 and induces their rapid degradation (31, 32). Consistently, SM treatment also inhibited SeV-induced ubiquitination of TRAF3 (Fig. 6*B*). Taken together, these results suggest that cIAP1 and cIAP2 mediate virus-triggered ubiquitination of TRAF3 at the mitochondria.

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 inhib-

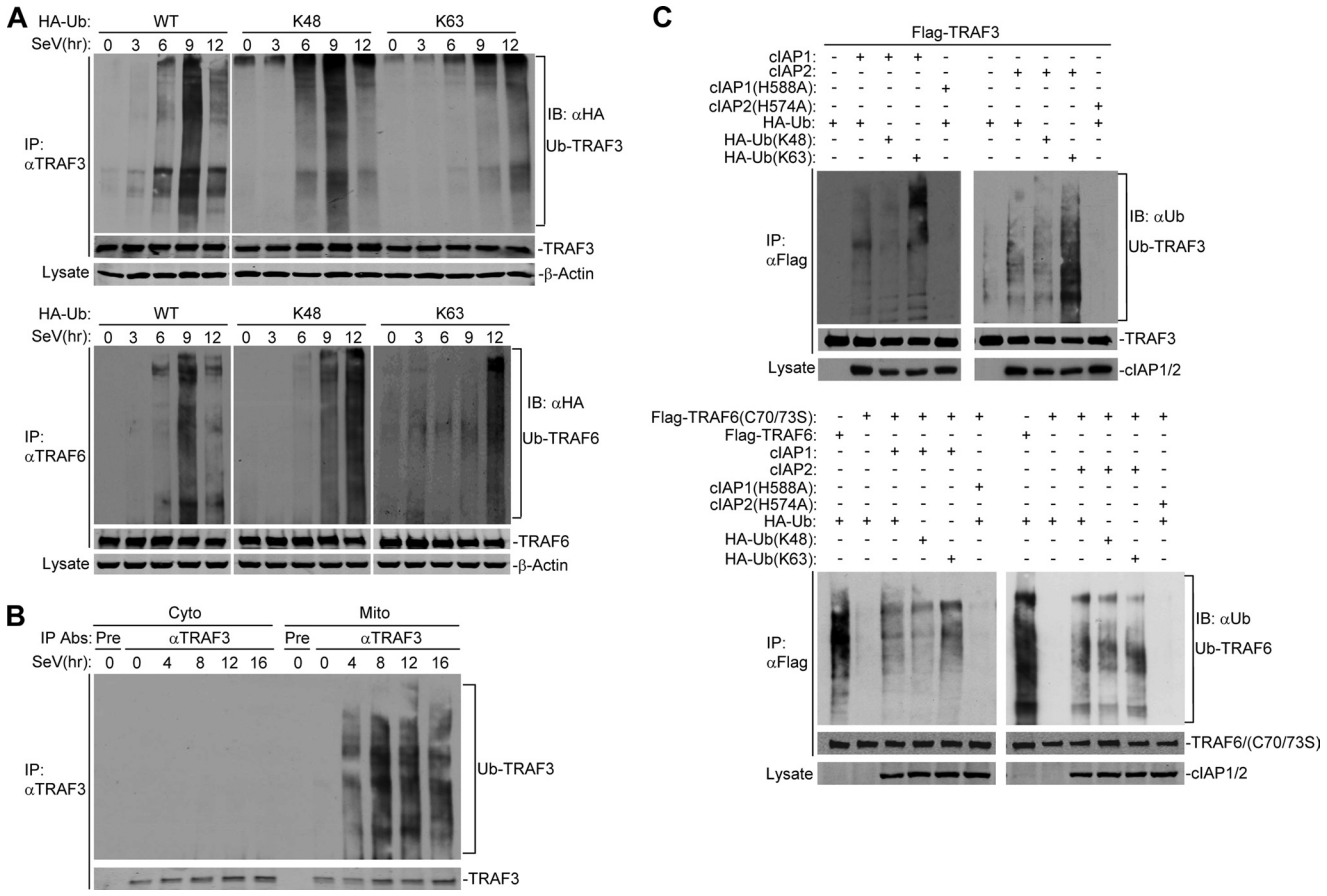

FIGURE 5. **Virus-triggered TRAF3 and TRAF6 ubiquitination.** *A*, SeV induced both Lys-48- and Lys-63-linked ubiquitination of endogenous TRAF3 and TRAF6. 293 cells (3×10^7) were transfected with HA-tagged wild type (*WT*), Lys-48-only or Lys-63-only ubiquitin plasmids (5 μg each). 24 h after transfection, cells were infected with SeV for the indicated time points. The cell lysates were immunoprecipitated with anti-TRAF3 or anti-TRAF6 as indicated. The immunoprecipitates were analyzed by immunoblots with anti-HA, anti-TARF3, or anti-TRAF6 as indicated (*upper panels*). A fraction of lysate was taken for immunoblot analysis with anti-β-actin (*lower panel*). *B*, SeV-induced ubiquitination of TRAF3 located at the mitochondria. 293 cells (3×10^7) were transfected with HA-Ub plasmids (5 μg each) and infected with SeV as in *A*. Cells were fractionated into cytosolic (*Cyto*) and mitochondrial (*Mito*) fractions. Lysates of the fractions were immunoprecipitated with anti-TRAF3 or preimmune serum (*Pre*) as indicated. The immunoprecipitates were analyzed by immunoblots with anti-ubiquitin (*upper panel*) or anti-TRAF3 (*lower panel*). *C*, cIAP1 and cIAP2 catalyze both Lys-48- and Lys-63-linked ubiquitination of TRAF3 and TRAF6. 293 cells (2×10^6) were transfected with the indicated plasmids. 20 h after transfection, cell lysates were immunoprecipitated with anti-Flag. The immunoprecipitates were analyzed by immunoblots with anti-ubiquitin, anti-TRAF3, or anti-TRAF6 as indicated. The levels of cIAP1 and cIAP2 in the lysates were examined by immunoblots with anti-cIAP1 and anti-cIAP2, respectively.

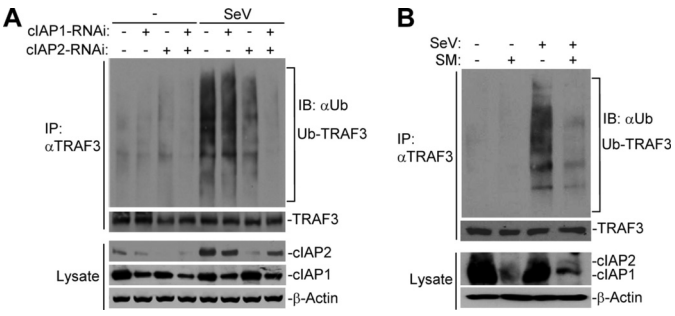

FIGURE 6. **cIAP1 and cIAP2 mediate virus-triggered TRAF3 ubiquitination.** *A*, effects of cIAP1 and cIAP2 RNAi on SeV-induced ubiquitination of endogenous TRAF3. 293 cells (5×10^7) were transfected with the indicated RNAi plasmids. 12 h later cells were selected with puromycin (1 μg/ml) for 24 h and then infected with SeV or left uninfected for 8 h. The cell lysates were immunoprecipitated with anti-TRAF3. The immunoprecipitates were analyzed by immunoblots with anti-ubiquitin and anti-TRAF3 as indicated (*upper panels*). The levels of cIAP1/2 and β-actin were examined by immunoblots with the indicated antibodies (*lower panels*). *B*, effects of SM on SeV-induced ubiquitination of endogenous TRAF3. 293 cells (5×10^7) were treated with PBS or SM (100 nM) for 12 h. The cells were then infected with SeV or left uninfected for 8 h. Immunoprecipitation and immunoblotting analysis were similarly performed as in *A*.

ited 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-

uitination 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, IKKϵ, 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.

Acknowledgments—We thank Dr. Xiaodong Wang for the Smac mimetic reagent and members of our laboratory for discussions. The authors declare no conflicts of interest.

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