ORIGINAL ARTICLE

Negative feedback regulation of cellular antiviral signaling by RBCK1-mediated degradation of IRF3

Min Zhang[1], Yang Tian[1], Rui-Peng Wang[1], Dong Gao[1], Yan Zhang[1], Fei-Ci Diao[1], Dan-Ying Chen[1], Zhong-He Zhai[1], Hong-Bing Shu[2]

[1]College of Life Sciences, Peking University, Beijing 100871, China; [2]College of Life Sciences, Wuhan University, Wuhan 430072, China

Viral infection causes host cells to produce type I interferons (IFNs), which are critically involved in viral clearance. Previous studies have demonstrated that activation of the transcription factor interferon regulatory factor (IRF)3 is essential for virus-triggered induction of type I IFNs. Here we show that the E3 ubiquitin ligase RBCC protein interacting with PKC1 (RBCK1) catalyzes the ubiquitination and degradation of IRF3. Overexpression of RBCK1 negatively regulates Sendai virus-triggered induction of type I IFNs, while knockdown of RBCK1 has the opposite effect. Plaque assays consistently demonstrate that RBCK1 negatively regulates the cellular antiviral response. Furthermore, viral infection leads to induction of RBCK1 and subsequent degradation of IRF3. These findings suggest that the cellular antiviral response is controlled by a negative feedback regulatory mechanism involving RBCK1-mediated ubiquitination and degradation of IRF3.

Keywords: RBCK1, IRF3, antiviral response, type I IFNs, feedback regulation, E3 ligase

Introduction

Host cells respond to viral infection by producing and secreting a family of cytokines called type I interferons (IFNs), including IFNβ and IFNα family members. These cytokines induce the expression of numerous downstream proteins that are collectively involved in the inhibition of viral replication [1-4]. Transcriptional regulation of the type I IFN genes requires the assembly of an enhanceosome that contains several transcription factors. For example, the IFNβ gene promoter contains a κB site recognized by nuclear factor kappa B (NF-κB), a site for ATF-2/c-Jun and two IFN-stimulated response elements (ISREs) recognized by phosphorylated interferon regulatory factor (IRF)3 or IRF7. Upon viral infection, these transcription factors are activated and cooperatively assemble into an enhanceosome on the IFNβ gene promoter, thereby activating the transcription of IFNβ [5, 6].

The signaling pathways leading to the induction of type I IFNs have begun to emerge. Viral infection results in the accumulation of single- or double-stranded viral RNAs in the cytoplasm. The viral RNAs are recognized by two caspase recruitment domain (CARD)-containing RNA helicase proteins, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated protein-5 (MDA5), which signal through a CARD-containing adapter protein called VISA by our group and MAVS, IPS-1, or Cardif by others [7-13]. Various studies have demonstrated that VISA plays a critical role in virus-induced NF-κB activation, although it is controversial how this is achieved. On the other hand, it is widely accepted that VISA recruits TANK-binding kinase 1 (TBK1) and IRF3 to the RIG-I/MDA5 signaling complexes, in which IRF3 is phosphorylated by TBK1. Phosphorylated IRF3 dimerizes, forms a complex with CBP/p300, and translocates into the nucleus to participate in the transcriptional activation of the type I IFN genes [14-17].

Type I IFNs are undetectable in cells under physi-

Correspondence: Hong-Bing Shu[a], Dan-Ying Chen[b]
[a]Tel: +86-27-68753795; Fax:+86-27-68753780
E-mail: shuh@whu.edu.cn
[b]Tel: +86-10-62757924; Fax: +86-10-62757924
E-mail: dychen@pku.edu.cn

Abbreviations: NF-κB (nuclear factor kappa B); IRF (interferon regulatory factor); ISRE (IFN-stimulated response elements); CARD (caspase recruitment domain); TBK1 (TANK-binding kinase 1); IKKε (inhibitor of nuclear factor κB kinase subunit epsilon); RIG-I (retinoic acid-inducible gene I; MDA5 (melanoma differentiation associated protein-5); SeV (Sendai virus); RBCK1 (RBCC protein interacting with PKC1)

ological conditions, but are promptly induced upon viral infection. To prevent autonomous production of type I IFNs or overproduction of these cytokines following viral infection, which causes a pathological immune response, the cell has evolved distinct strategies to tightly regulate their expression. It has been shown that LGP2, a helicase protein lacking CARDs at its N-terminus, acts as a negative regulator by sequestering viral RNA from RIG-I [18-21]. The cellular protein DAK is associated with MDA5, and specifically inhibits MDA5-mediated, but not RIG-I-mediated, signaling [22]. The coiled-coil protein SIKE is associated with TBK1/inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) and keeps these kinases inactive under physiological conditions [23].

Recent studies have demonstrated that post-translational modification by ubiquitin plays an important role in the regulation of virus-triggered type I IFN signaling. The dual-function deubiquitinating enzyme and ubiquitin ligase A20 negatively regulate RIG-I-induced antiviral signaling through an unidentified mechanism [24]. DUBA, a deubiquitinating enzyme, turns off expression of type I IFNs by deubiquitinating TRAF3, a signaling component in virus-triggered type I IFN signaling pathways [25]. The E3 ubiquitin ligase RNF125 targets RIG-I, MDA5 and VISA for degradation, and therefore negatively regulates type I IFN production [26]. In contrast, a recent study suggests that Lys 63-linked ubiquitination of RIG-I by the E3 ubiquitin ligase TRIM25 is crucial for the RIG-I-mediated cellular antiviral response [27].

RBCC protein interacting with PKC1 (RBCK1), also called HOIL-1 [28-30], XAP3 [31], or UIP28 [32], contains an N-terminal ubiquitin-like domain (UBL) and a C-terminal RING-IBR-RING (RBR) signature structure. It has been shown that RBCK1 is an E3 ubiquitin ligase for proteins involved in iron metabolism and for SOCS6 [28-30, 33]. Recently, we showed that RBCK1 negatively regulates TNF- and IL-1-induced NF-κB activation by targeting TAB2/3 for degradation [34]. In this report, we demonstrate that RBCK1 catalyzes the ubiquitination and degradation of IRF3, and negatively regulates virus-triggered type I IFN production and cellular antiviral response. In addition, we found that viral infection leads to induction of RBCK1 and subsequent degradation of IRF3. These findings suggest that the cellular antiviral response is controlled by a feedback negative regulatory mechanism involving RBCK1-mediated ubiquitination and degradation of IRF3.

Results

RBCK1 suppresses virus-triggered transcriptional activation of IFNβ and IFNα4

Previously, we demonstrated that RBCK1 negatively regulates TNF- and IL1-induced NF-κB activation by targeting TAB2/3 for degradation [34]. Since virus-triggered induction of type I IFNs involves NF-κB activation, we examined whether RBCK1 regulates virus-triggered signaling pathways. Reporter assays indicated that overexpression of RBCK1 dramatically inhibited Sendai virus (SeV)-induced transcriptional activation of IFNβ and IFNα4 gene promoters (Figure 1A). Furthermore, overexpression of RBCK1 inhibited SeV-induced expression of endogenous IFNβ and IFNα4 (Figure 1B).

To determine whether RBCK1 plays a negative regulatory role in virus-triggered type I IFN signaling under physiological conditions, we determined the effect of knockdown of RBCK1 by RNAi. We used two RBCK1 RNAi constructs in this study; one (#4) significantly inhibited expression of RBCK1, while the other (#1) had no detectable effect (Figure 1C). In reporter assays, knockdown of RBCK1 by the #4 RNAi construct was sufficient to activate the IFNβ and IFNα4 promoters. In the same experiments, knockdown of RBCK1 potentiated SeV-induced activation of the IFNβ and IFNα4 promoters (Figure 1D). In addition, knockdown of RBCK1 also induced or potentiated expression of endogenous IFNβ and IFNα4 in the absence or presence of viral stimulation (Figure 1E). These data suggest that RBCK1 is a physiological suppressor of virus-triggered type I IFN signaling.

RBCK1 negatively regulates the cellular antiviral response

Since RBCK1 is a physiological suppressor of virus-triggered type I IFN signaling, we determined whether RBCK1 plays a role in the cellular antiviral response. Previously, it was shown that wild-type VSV induces little expression of type I IFNs in 293 cells, while overexpression of RIG-I-N, MDA5-N, VISA, or TBK1 can induce transcription of type I IFNs, and causes a cellular antiviral response [7-13, 16, 17]. Accordingly, we tested whether RBCK1 affects the cellular antiviral response mediated by these signaling proteins in 293 cells. Using plaque assays, we found that overexpression of RBCK1 was able to significantly antagonize the inhibitory effect on viral replication mediated by RIG-I-N, MDA5-N, VISA, or TBK1 (Figure 2A). Consistently, knockdown of RBCK1 inhibited viral replication (Figure 2B). These results suggest that RBCK1 acts as a physiological inhibitor of the cellular antiviral response.

RBCK1 negatively regulates virus-triggered IFN signaling by destabilizing IRF3

To determine the molecular mechanisms responsible for RBCK1-mediated inhibition of virus-triggered type I IFN
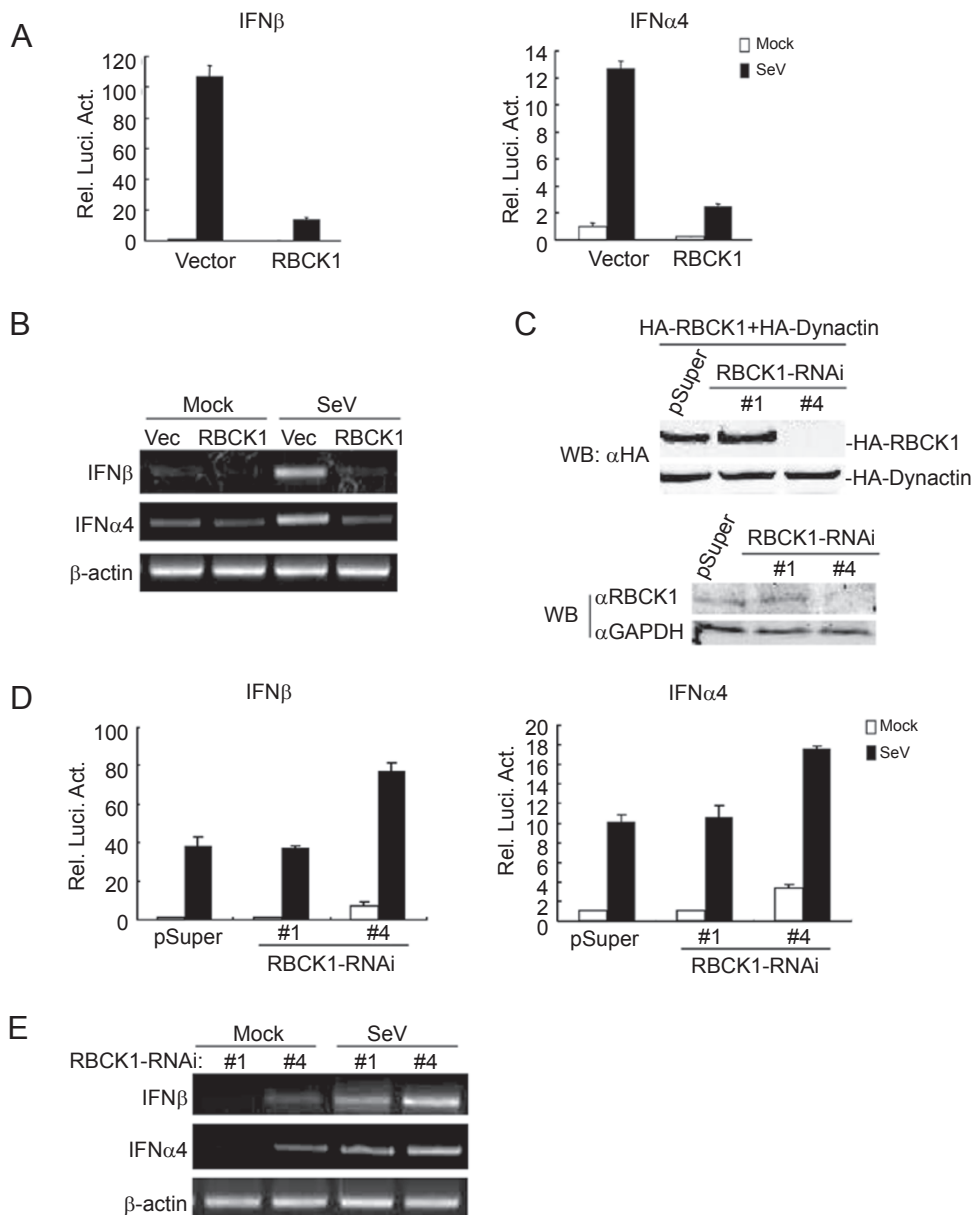
Figure 1 RBCK1 regulates SeV-triggered type I IFN signaling. **(A)** RBCK1 inhibits SeV-induced activation of IFN and IFNα4 promoters. The 293 cells (~5×10^4) were transfected with IFNβ or IFNα4 promoter luciferase plasmid (50 ng), pRL-TK Renilla luciferase plasmid (50 ng), and an expression vector for Flag-RBCK1 (0.1 μg) or an empty vector (0.1 μg). At 12 h after transfection, cells were infected with SeV (filled bars) or left uninfected (open bars) for 12 h before luciferase assays were performed. **(B)** RBCK1 inhibits SeV-induced expression of endogenous IFNβ and IFNα4. The 293 cells (2×10^5) were transfected with an expression vector for Flag-RBCK1 (0.5 μg) or an empty vector (0.5 μg). At 12 h after transfection, cells were infected with SeV or left uninfected for 12 h before RT-PCR analysis of the indicated mRNAs. **(C)** Effects of RBCK1 RNAi plasmids on the expression of overexpressed and endogenous RBCK1. The 293 cells (2×10^5) were transfected with HA-tagged RBCK1 and Dynactin (upper panels) or empty control plasmid (lower panels) (1 μg each), together with pSuper control, RBCK1-RNAi-#1 or RBCK1-RNAi-#4 plasmid (2 μg each) as indicated. At 48 h after transfection, cell lysates were analyzed by Western blot with anti-HA (upper panels), anti-RBCK1, or anti-GAPDH (lower panels) antibody. **(D)** Knockdown of RBCK1 potentiates SeV-triggered activation of IFNβ and IFNα4 promoters in 293 cells. The 293 cells (~5×10^4) were transfected with the IFNβ or IFNα4 promoter reporter plasmids (50 ng) and the indicated RBCK1 RNAi plasmid (1 μg). At 36 h after transfection, cells were infected with SeV or left uninfected for 12 h before luciferase assays. **(E)** knockdown of RBCK1 increased SeV-triggered expression of endogenous IFNβ and IFNα4. The 293 cells (2×10^5) were transfected with a control or the indicated RBCK1 RNAi plasmid (2 μg each). Thirty-six hours after transfection, cells were infected with SeV or left uninfected for 12 h before RT-PCR analysis of the expression of the indicated mRNAs.
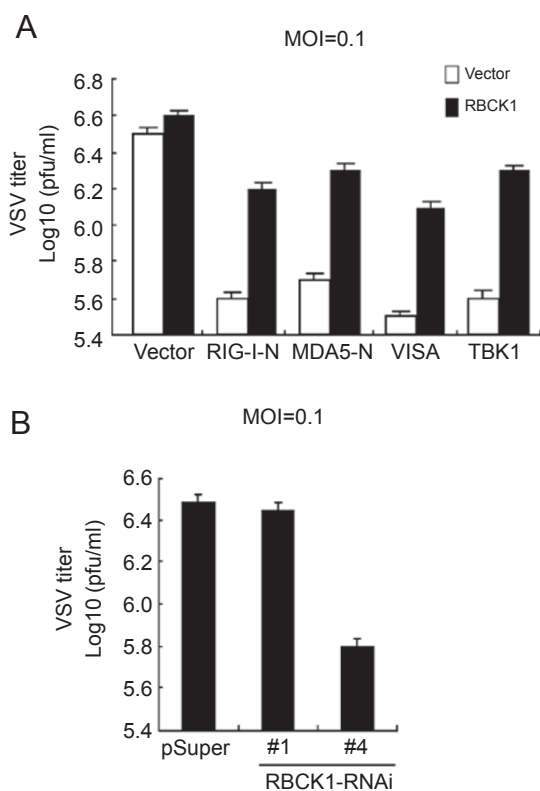
A

MOI=0.1

□ Vector
■ RBCK1

B

MOI=0.1

Figure 2 Effect of overexpression or knockdown of RBCK1 on the cellular antiviral response. **(A)** Overexpression of RBCK1 inhibits RIG-I-N-, MDA5-N-, VISA- or TBK1-mediated cellular antiviral response. The 293 cells (2×10^5) were transfected with the indicated expression plasmids (1 µg each). Twenty-four hours after transfection, cells were infected with VSV [multiplicity of infection (MOI) = 0.1], and supernatants were harvested at 12 h postinfection and analyzed for VSV production by standard plaque assays. **(B)** Knockdown of RBCK1 enhances the cellular antiviral response. The 293 cells (2×10^5) were transfected with a control or the indicated RBCK1 RNAi plasmid (2 µg each). Thirty-six hours after transfection, cells were infected with VSV (MOI = 0.1), and supernatants were harvested at 12 h postinfection for plaque assays.

signaling, we examined whether RBCK1 inhibits virus-triggered NF-κB and IRF3 activation. As shown in Figure 3A, RBCK1 significantly inhibited both SeV-induced NF-κB and ISRE activation. Previously, we demonstrated that RBCK1 targets TAB2/3 for degradation and therefore inhibits TNF- and IL1-induced NF-κB activation [34]. Since TAK1, a kinase associated with TAB2/3, is involved in SeV-induced NF-κB [11], it is possible that RBCK1 inhibits SeV-induced NF-κB activation by targeting TAB2/3. Therefore, we focused on the mechanism of RBCK1-mediated inhibition of ISRE. To determine which signaling components in the virus-triggered IRF3 activation pathway are targeted by RBCK1, we determined the effect

of RBCK1 on ISRE activation mediated by the involved signaling components. In reporter assays, RBCK1 had a significant inhibitory effect on ISRE activation triggered by overexpression of RIG-I-N, MDA5-N, VISA, TBK1, and IRF3, but not IRF7 (Figure 3B), suggesting that RBCK1 functions specifically at or downstream of IRF3.

We next sought to identify the relationship between RBCK1 and IRF3. Interestingly, the protein level of IRF3, but not IRF7, was dramatically down-regulated when it was coexpressed with RBCK1 (Figure 3C). In addition, overexpression of RBCK1 caused down-regulation of endogenous IRF3, and this effect was reversed by MG132, a proteasome inhibitor (Figure 3D). Moreover, knockdown of RBCK1 by RNAi caused an increased level of endogenous IRF3 (Figure 3E). Taken together, these data suggest that RBCK1 targets IRF3 for degradation by the proteasomes.

RBCK1 physically interacts with and ubiquitinates IRF3

Since RBCK1 targets IRF3 for degradation in a proteasome-dependent manner, we determined whether RBCK physically interacts with and ubiquitinates IRF3. In transient transfection and coimmunoprecipitation experiments, RBCK1 interacted with IRF3 in the presence of MG132 (Figure 4A). Moreover, the N-terminal UBL domain of RBCK1, RBCK1 (1-220), was sufficient for its interaction with IRF3, whereas an interaction between IRF3 and the C-terminal RBR domain of RBCK1, RBCK1 (221-510), was undetectable (Figure 4B). In reporter assays, RBCK1 (221-510) inhibited SeV-induced ISRE activation as potently as did full-length RBCK1, while RBCK1 (1-220) inhibited SeV-induced ISRE activation to a lesser degree (Figure 4C). Taken together, these data suggest that RBCK1 physically interacts with IRF3 via its UBL domain, and destabilizes IRF3 via the RBR domain.

Since RBCK1 is an E3 ubiquitin ligase and causes IRF3 degradation, we determined whether RBCK1 ubiquitinates IRF3. We transfected 293 cells with expression plasmids for Flag-IRF3, HA-ubiquitin, and HA-RBCK1. Coimmunoprecipitation and western blot analysis indicated that RBCK1 significantly polyubiquitinated IRF3 (Figure 4D).

RBCK1 is up-regulated following viral infection

Having established that RBCK1 negatively regulates innate antiviral signaling by targeting IRF3 for degradation, we next examined whether viral infection affects RBCK1 expression. We found that endogenous RBCK1 was up-regulated following SeV infection in a time-dependent manner (Figure 5A). Interestingly, SeV-induced up-regulation of RBCK1 was accompanied by a temporal reduction of endogenous IRF3 (Figure 5A). Knockdown of RBCK1 by RNAi rescued SeV-induced IRF3 down-regulation (Figure 5B). These observations suggest that RBCK1
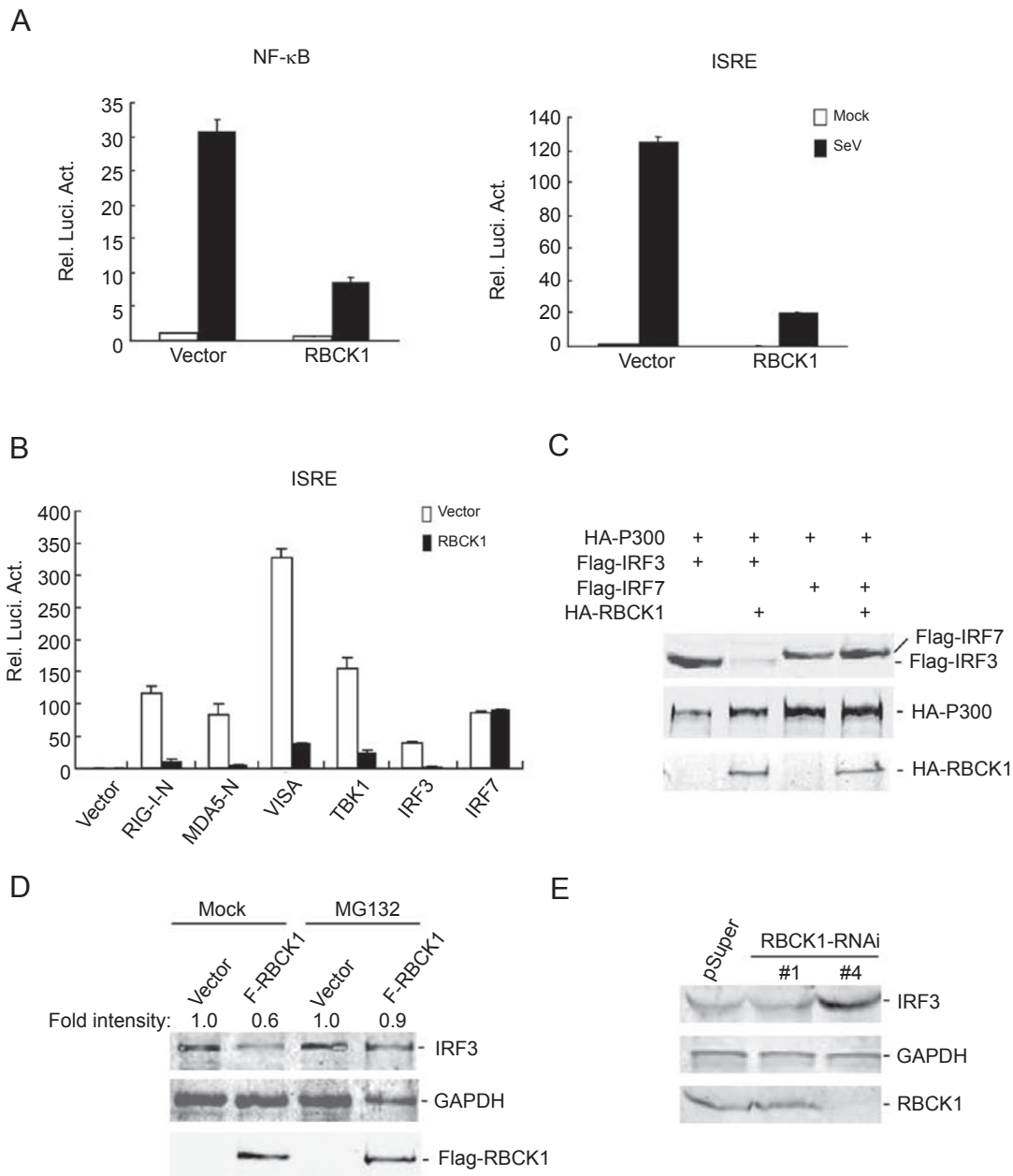
Figure 3 RBCK1 inhibits virus-triggered IFN signaling by targeting IRF3 for degradation. **(A)** RBCK1 inhibits SeV-triggered NF-κB and ISRE activation. The 293 cells (~5×10^4) were transfected with NF-κB or ISRE luciferase plasmid (50 ng), pRL-TK Renilla luciferase plasmid (50 ng), and an expression vector for Flag-RBCK1 (0.1 µg) or an empty vector (0.1 µg). At 12 h after transfection, cells were infected with SeV (filled bars) or left uninfected (open bars) for 12 h before luciferase assays were performed. **(B)** RBCK1 inhibits ISRE activation mediated by RIG-I-N, MDA5-N, VISA, TBK1, and IRF3, but not IRF7. The 293 cells (~5×10^4) were transfected with ISRE luciferase plasmid (50 ng), pRL-TK Renilla luciferase plasmid (50 ng), and the indicated plasmids (0.1 µg each). At 24 h after transfection, luciferase assays were performed. **(C)** RBCK1 specifically destabilizes IRF3. The 293 cells (2×10^5) were transfected with the indicated expression plasmids (1 µg each). At 24 h after transfection, cell lysates were analyzed by western blot with anti-Flag (upper panel) or anti-HA (middle and lower panels) antibody. **(D)** RBCK1 causes degradation of endogenous IRF3 in a proteasome-dependent manner. The 293 cells (2×10^5) were transfected with an expression vector for Flag-RBCK1 or an empty vector (1 µg each). At 24 h after transfection, cells were treated with MG132 (10 µM) or left untreated for 6 h before Western blot analysis was performed with anti-IRF3 (upper panel), anti-GAPDH (middle panel), or anti-Flag (lower panel). **(E)** Knockdown of RBCK1 up-regulates endogenous IRF3. The 293 cells were transfected as in Figure 1C. At 48 h after transfection, cell lysates were analyzed by Western blot with anti-IRF3 (upper panel), anti-GAPDH (middle panel), or anti-RBCK1 (lower panel) antibody.
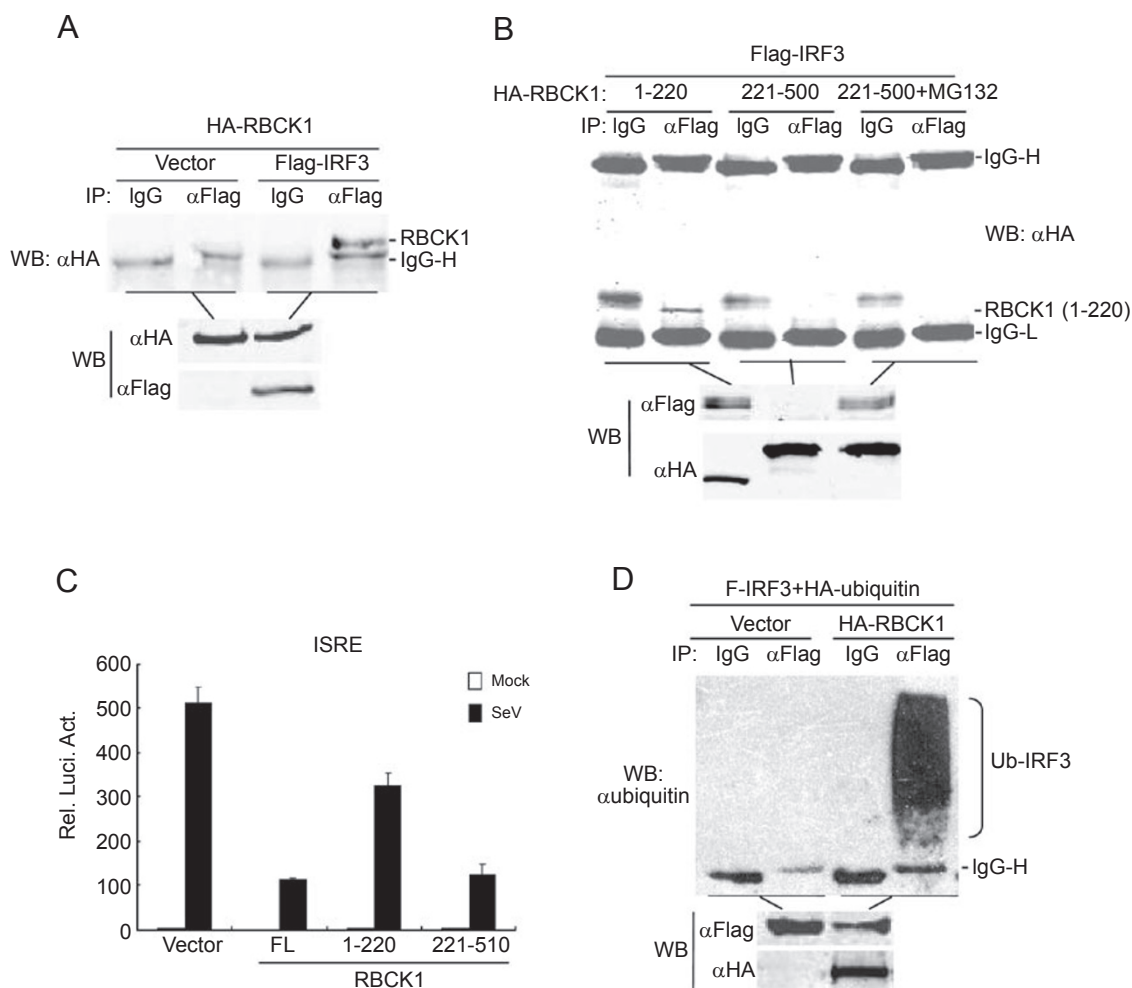
A

B

C

D

Figure 4 RBCK1 interacts with and ubiquitinates IRF3. **(A)** RBCK1 interacts with IRF3. The 293 cells (2×10^6) were transfected with the indicated plasmids (3 µg each). At 18 h after transfection, cells were treated with MG132 (10 µM) for 6 h before cell lysates were immunoprecipitated with anti-Flag antibody (αFlag) or control mouse IgG (mIgG). The immunoprecipitates were analyzed by western blot with anti-HA antibody (upper panel). Expression of the transfected proteins was analyzed by western blot with anti-Flag or anti-HA antibody (middle and lower panels). **(B)** Domain mapping of the interaction between RBCK1 and IRF3. The 293 cells (2×10^6) were transfected with the indicated plasmids (3 µg each) and coimmunoprecipitation experiments were performed as in (A). **(C)** Effects of RBCK1 mutants on SeV-triggered ISRE activation. The 293 cells ($\sim 5 \times 10^4$) were transfected with ISRE luciferase plasmid (50 ng), pRL-TK Renilla luciferase plasmid (50 ng), and the indicated plasmids (0.1 µg). At 12 h after transfection, cells were infected with SeV (filled bars) or left uninfected (open bars) for 12 h before luciferase assays were performed. **(D)** RBCK1 ubiquitinates IRF3. The 293 cells (2×10^6) were transfected with expression plasmids for Flag-IRF3, HA-ubiquitin, HA-RBCK1, or control plasmid (3 µg each). At 18 h after transfection, cells were treated with MG132 (10 µM) for 6 h. Cell lysates were immunoprecipitated with anti-Flag antibody (αFlag) or control mouse IgG (mIgG). The immunoprecipitates were analyzed by western blot with anti-ubiquitin antibody (upper panel). Expression of the transfected proteins was analyzed by western blot with anti-Flag or anti-HA antibody (middle and lower panels).

regulates the innate antiviral response through a negative feedback mechanism.

Discussion

Viral infection results in transcriptional induction of type I IFNs and other cytokines that have inhibitory effects on viral replication in infected and neighboring uninfected cells. However, uncontrolled excessive production of type I IFNs significantly contributes to chronic inflammation and autoimmune diseases, underscoring the importance of negative regulation of type I IFN [35-38]. The host innate immune system has developed distinct strategies to ensure proper production of type I IFNs following viral infection
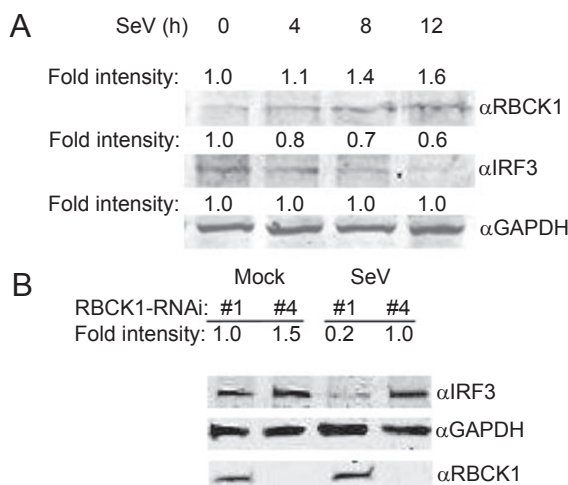
A

SeV (h)	0	4	8	12	
Fold intensity:	1.0	1.1	1.4	1.6	αRBCK1
Fold intensity:	1.0	0.8	0.7	0.6	αIRF3
Fold intensity:	1.0	1.0	1.0	1.0	αGAPDH

B

	Mock		SeV		
RBCK1-RNAi:	#1	#4	#1	#4	
Fold intensity:	1.0	1.5	0.2	1.0	
					αIRF3
					αGAPDH
					αRBCK1

Figure 5 RBCK1 mediates SeV-induced IRF3 down-regulation. **(A)** Expression of RBCK1 is induced by SeV. The 293 cells were infected by SeV for the indicated times before cell lysates were analyzed by Western blot with anti-RBCK1 (upper panel), anti-IRF3 (middle panel), or anti-GAPDH (lower panel). **(B)** Knockdown of RBCK1 rescues SeV-induced down-regulation of IRF3. The 293 cells were transfected with the indicated RBCK1 RNAi plasmids. At 36 h after transfection, cells were infected with SeV for 12 h or left untreated. Cell lysates were analyzed by Western blot with the indicated antibodies.

[18-26]. In the present work, we identified the E3 ubiquitin ligase RBCK1 as a negative feedback regulator of the cellular antiviral response.

Our results demonstrated that overexpression of RBCK1 significantly inhibits SeV-induced expression of type I IFNs, while knockdown of RBCK1 induces or potentiates SeV-induced expression of type I IFNs (Figure 1). In analyzing the target of RBCK1 in virus-induced type I IFN signaling pathways, we found that RBCK1 interacts with and ubiquitinates IRF3 and causes its degradation through a proteasome-dependent manner, while knockdown of RBCK1 by RNAi causes an increased level of IRF3 (Figures 3 and 4). Although IRF3 shares a similar structure with IRF7, RBCK1 destabilizes IRF3 but not IRF7, suggesting that RBCK1 specifically regulates IRF3-dependent signaling. Domain mapping experiments suggest that the N-terminal UBL domain of RBCK1 is sufficient for its interaction with IRF3, whereas the C-terminal RBR domain is responsible for degrading IRF3 and inhibiting SeV-induced ISRE activation (Figure 4B). Although the N-terminal UBL domain lacks E3 ubiquitin ligase activity, it weakly inhibited SeV-induced ISRE activation in reporter assays (Figure 4C). This effect is probably caused by its recruitment of endogenous RBCK1 to IRF3. The C-terminal RBR domain of RBCK1, RBCK1 (221-510), potently induced degradation of IRF3 and inhibited SeV-induced ISRE ac-

tivation, even though it did not interact with IRF3 (Figure 4B and 4C). The simplest explanation for this observation is that our coimmunoprecipitation experiments were not sensitive enough to detect their interaction.

Our studies indicate that viral infection induces expression of RBCK1, which is accompanied by down-regulation of IRF3. In addition, virus-induced IRF3 down-regulation is rescued by knockdown of RBCK1 (Figure 5). Taken together, these data suggest that the E3 ubiquitin ligase RBCK1 targets IRF3 for degradation and regulates virus-induced type I IFN signaling through a negative feedback regulatory mechanism.

Previously, various studies have demonstrated that IRF3 degradation following virus infection plays a significant role in the termination of the IRF3-dependent cellular antiviral response [14, 39-44]. These studies have identified several cellular and viral proteins that are involved in IRF3 ubiquitination and degradation, including an unidentified Cullin-based ubiquitin ligase [41], the peptidyl-prolyl isomerase Pin1 [42], ICP0 of herpesvirus 1 [39, 40, 43], and NSP1 of rotavirus [44]. However, a bona fide cellular E3 ubiquitin ligase for IRF3 is unknown. Our discovery that RBCK1 is a virus-inducible E3 ubiquitin ligase for IRF3 ubiquitination and degradation resolves the molecular mechanism accounting for IRF3 degradation-dependent control of excessive cellular antiviral response.

Materials and Methods

Reagents and antibodies

Mouse monoclonal antibodies against Flag (M2) and HA epitopes (Sigma, St Louis, USA), rabbit polyclonal antibodies against IRF3 (SC-9082) and ubiquitin (SC-9133) (Santa Cruz Biotechnology, Santa Cruz, USA), VSV (Dr Hong-Kui Deng, Peking University, Beijing, China) and SeV (Dr Cong-Yi Zheng, Wuhan University, Wuhan, China) were obtained from the indicated sources. Mouse anti-RBCK1 antiserum was described previously [34].

Constructs

Mammalian expression plasmids for Flag-RIG-I, Flag-MDA5, Flag-VISA, Flag-TBK1, Flag-IRF3, Flag-IRF7, Flag- or HA-tagged RBCK1 and RBCK1 deletion mutants were described previously [11, 22, 23]. Mammalian expression plasmid for HA-P300 was provided by Dr David Livingston (Dana Farber Cancer Institute, Boston, USA). ISRE-luciferase reporter plasmid was purchased from Stratagene (West Cedar Creek, USA). NF-κB and IFNβ promoter luciferase reporter plasmids were described previously [11]. IFNα4-luciferase reporter plasmid was provided by Dr Zheng-Fan, Jiang (Peking University, Peking, China).

Transfection and reporter gene assays

293 cells (~5×10^4) were seeded on 24-well dishes and transfected the following day by standard calcium phosphate precipitation. To normalize for transfection efficiency, 0.1 mg of pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Ap-

proximately 20 h after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (Promega, Madison, WI). Firefly luciferase activities were normalized based on *Renilla* luciferase activities. All reporter gene assays were repeated at least three times. Data shown are average values±s.d. from one representative experiment.

RT-PCR

Total RNA was isolated from 293 cells using Trizol reagent (Tiangen Bio-Tech, China). cDNA was made from total RNA using M-MLV (Promega, USA) according to the manufacturer's instructions. Gene-specific primer sequences were as follows.

IFN-β: 5'-CAC GAC AGC TCT TTC CAT GA-3' (forward), 5'-AGC CAG TGC TCG ATG AAT CT-3' (reverse);

IFNα4: 5'-CCC GAG GAG GAG TTT GAT-3' (forward), 5'-TTC CAG GTC ATT CAG TTG C-3' (reverse);

β-actin: 5'-GTC GTC GAC AAC GGC TCC GGC ATG-3' (forward), 5'-ATT GTA GAA GGT GTG GTG CCA GAT-3' (reverse).

RNAi experiments

The experiments were performed as described [34].

VSV plaque assay

293 cells grown in media containing 1% FBS were incubated with VSV at MOI 0.1 for 1 h before replacement with the complete media containing 10% FBS. After 24 h, the supernatant was harvested and diluted to infect confluent BHK21 cells cultured on 24-well dishes. At 1 h postinfection, the supernatant was removed and 2% methylcellulose was overlaid. On day 3 postinfection, the overlay was removed and cells were fixed with 0.5% glutaraldehyde for 30 min and stained with 1% crystal violet in 70% methanol for 15 min. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titer as Log10 (pfu/ml).

Coimmunoprecipitation and western blot analysis

For transient transfection and coimmunoprecipitation experiments, 293 cells (~1×10^6) were transfected for 24 h. The transfected cells were lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, a 0.4 ml aliquot of lysate was incubated for 2 h with 0.5 μg of the indicated antibody or control IgG and 25 μl of 1:1 slurry of GammaBind G Plus-Sepharose (GE Healthcare). The Sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were analyzed by western blots as described [11].

Acknowledgments

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