Ubiquitin-Specific Proteases 25 Negatively Regulates Virus-Induced Type I Interferon Signaling

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Abstract

Ubiquitination and deubiquitination have emerged as critical regulatory processes in the virus-triggered type I interferon (IFN) induction pathway. In this study, we carried out a targeted siRNA screen of 54 ubiquitin-specific proteases (USPs) and identified USP25 as a negative regulator of the virus-triggered type I IFN signaling pathway. Overexpression of USP25 inhibited virus-induced activation of IFN-β, interferon regulation factor 3 (IRF3) and nuclear factor-kappa B (NF-κB), as well as the phosphorylation of IRF3 and NF-κB subunit p65. Furthermore, Knockdown of USP25 potentiated virus-induced induction of the IFN-β. In addition, detailed analysis demonstrated that USP25 cleaved lysine 48- and lysine 63-linked polyubiquitin chains in vitro and in vivo, and its deubiquitinating enzyme (DUB) activity, were dependent on a cysteine residue (Cys178) and a histidine residue (His607). USP25 mutants lacking DUB activity lost the ability to block virus-induced type I IFN to some degree. Mechanistically, USP25 deubiquitinated retinoic acid-inducible gene I (RIG-I), tumornecrosis factor (TNF) receptor-associated factor 2 (TRAF2), and TRAF6 to inhibit RIG-I-like receptor-mediated IFN signaling. Our findings suggest that USP25 is a novel DUB negatively regulating virus-induced type I IFN production.

Introduction

Innate immune responses are activated through host pattern recognition receptors (PRRs), which recognize molecular structures called pathogen-associated molecular patterns (PAMPs) that are structurally conserved within large groups of pathogens. Upon engagement of PAMPs, PRRs initiate signaling pathways, ultimately triggering the production of type I interferons (IFNs) [1]. The type I IFN signal is then transduced by the activation of Janus kinase (JAK) family protein kinases and the signal transducers and activators of transcription (STAT) family of proteins [2], resulting in the generation of the transcriptional activator, IFN-stimulated gene 3 (ISGF3). ISGF3 bind to their specific DNA sequences containing each common motif; namely, the IFN-stimulated response element (ISRE), and participates in IFN responses. IFN stimulation of promoters containing ISRE results in the transcriptional induction of a large number of IFN-stimulated genes (ISGs) to evoke antiviral activity [3].

Studies during the past decades have revealed a working model on virus-triggered type I IFN signaling. Recognition of virus-derived double-stranded RNA (dsRNA) and 5'-triposphorylated single-stranded RNA (5''pppssRNA) by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) in the cytoplasm lead to the production of type I IFN and inflammatory cytokines [4-6]. RIG-I/MDA5 is indispensable for type I IFN responses to many RNA viruses, including influenza A virus, mumps virus, measles virus and Sendai virus (SEV) [7,8]. After sensing cytoplasmic viral RNAs, RIG-I and/or MDA5 interacts with the caspase activation and recruitment domain (CARD)-containing protein IFN-beta promoter stimulator 1 (IPS-1, also known as MAVS/VISA/Cardif) via CARD-like domains on both the RNA sensors and IPS-1 [9-12]. In turn, the IPS-1 complex recruits the downstream adaptor proteins tumornecrosis factor (TNF) receptor-associated factor 2 (TRAF2), TRAF3 and TRAF6, and TANK-binding kinase 1 (TBK1) and I kappaB kinase (IKK) α/β, leading to activation of the critical transcription factors interferon regulation factor 3 (IRF3) and nuclear factor-kappa B.
Modification of proteins by the covalent ligation of ubiquitin is a well-reviewed process that has been found to be involved in a myriad of cellular functions [15,16] such as apoptosis [17], DNA repair, cell cycle regulation [18], proteasome-dependent protein degradation [19], kinase activation and signal transduction [20-22]. Multiple ubiquitin ligases and ubiquitin-binding scaffold proteins contribute to positive regulation of the antiviral innate immune response, such as RIG-I, TRAF2, TRAF6 and TBK1. Post-translational modification of proteins by the covalent ligation of ubiquitin is a crucial regulatory mechanism, and the enzymes that catalyze these modifications have been the focus of many studies [23]. Deubiquitinizing enzymes (DUBs), which mediate the removal and processing of ubiquitin to recycle proteins, have a wide range of biological activity [24]. In recent years, the functions of DUBs in various pathways and signaling networks have been well reviewed. Accumulating evidence has revealed critical roles of deubiquitination in RIG-I-like receptor-mediated IFN signaling [25]. For example, deubiquitinating enzyme A (DUBA) selectively cleaves the lysine-63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream signaling complex containing TANK-binding kinase 1 (TAK1) [20]; the tumor suppressor cylindromatosis (CYLD) removes lysine-63-linked polyubiquitin chains from RIG-I, which inhibits the IRF3 signaling pathway [26]; A20 removes lysine-63-linked polyubiquitin chains from receptor interacting protein (RIP) [27].

Despite the importance of DUBs, the functions of DUBs during the antiviral innate immune response are largely unknown. To study the ubiquitin-specific proteases (USPs), as a subclass of the DUBs superfamily, we synthesized a collection of siRNA to suppress 54 human USPs, and used these siRNA to identify the enzymes regulating the antiviral immune response. By siRNA screening, we identified a cellular deubiquitinas, USP25, which regulates the virus-induced ISRE promoter. We found that USP25 processes K48-linked and K63-linked polyubiquitin in vitro and in vivo. Through its DUB activity, USP25 cleaves ubiquitin moieties from critical signaling proteins of the type I IFN signaling pathway, RIG-I, TRAF2, and TRAF6. Our findings present evidence that USP25 functions as a deubiquitinase that significantly inhibited virus-induced type I IFN signaling pathway.

Materials and Methods

Expression plasmids and small interfering RNA

The plasmids pNF-κB and pISRE-Luc were from Stratagene. (PRDIII-I) was kindly provided by S. Ludwig (Heinrich Heine University, Düsseldorf, Germany) [28]. Full-length hemagglutinin (HA)-tagged ubiquitin (Ub) mutants in which all but one Lys residue (HA-K48-Ub or HA-K63-Ub) was replaced with Arg were gifts from T. Ohta (St. Marianna University School of Medicine, Japan) [29]. pcDNA3.1-Flag-Ub and the IFS-1 expression vector were previously described [30,31]. The expression plasmids for wild-type (WT) RIG-I (pEF-Flag-RIG-I), its constitutively active mutant (pEF-Flag-RIG-I-N), and p125-Luc (IFN-β-Luc) were kindly provided by T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) [32]. The TRAF6 expression vector was a gift from Edward W. Harhaj (University of Miami School of Medicine, Miami, FL, USA). The expression plasmid for TRAF2 was constructed by PCR amplification of cDNA of TRAF2 (GenBank Accession No. NM021138) from HEK-293T (human embryonic kidney epithelial) cells, followed by cloning into the pCMV-Tag 2B vector (Stratagene).

The hemagglutinin (HA) or Myc epitope tag was amplified by PCR and cloned into the pCAGGS-MCS [33] vector to generate a pCAGGS-HA or pCAGGS-Myc plasmid with N-terminally HA or Myc tag, respectively. For construction of pCAGGS-HA-USP25 or pCAGGS-Myc-USP25, the cDNA fragment encoding the full-length USP25 (GenBank accession no. NM013396) was amplified by PCR from cDNA of HEK-293T cells and subcloned into the pCAGGS- HA or pCAGGS-Myc vector, respectively. Mutagenesis of individual amino acid residues (C178A and H607A) in USP25 were conducted using overlap extension PCR. Detailed sequences of the specific primers used are available upon request. All constructs were validated by DNA sequencing.

Double-stranded oligonucleotides corresponding to the target sequences of 54 USPs were synthesized by Sigma-Aldrich. The target sequences (5′ to 3′) of USP25 used in this study are listed in Table S1. The siRNA sequences (5′ to 3′) of other USPs used are available upon request.

Cell Culture and Virus

HEK-293T cells were cultured and maintained in RPMI-1640 (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO2 incubator. SEV was obtained from the Centre of Virus Resource and Information, Wuhan Institute of Virology, Chinese Academy of Sciences.

Transfection and reporter assay

Transient transfection was performed using Lipofectamine 2000 (Invitrogen). HEK-293T cells grown in 24-well plates were co-transfected with 0.1 μg of luciferase reporter plasmid (p125-Luc for IFN-β, (PRDIII-I)-Luc for IRF3, pNF-κB-Luc for NF-κB, and pISRE-Luc for ISRE), 0.02 μg of the Renilla luciferase construct phRL-TK (Promega) (for normalization of transfection efficiency) and various other expression plasmids or an empty control plasmid. In some experiments, cells were further infected or mock infected with SEV (10 hemagglutinating activity units/well) at 24 h after the initial co-transfection. Sixteen hours after SEV infection, cells were harvested. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega) according to the manufacturer's protocol. The relative luciferase activity was calculated by dividing the Firefly luciferase activity by the Renilla luciferase activity. All reporter assays were repeated at least three times. Data are presented as means ± standard deviation (SD).

Reverse transcription-PCR

Total RNA was isolated from HEK-293T cells using TRIzol reagent (Invitrogen). One microgram of this total RNA was
reverse transcribed to cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Toyobo, Japan), which (1 μL of 20 μL cDNA) was subsequently used in a SYBR green PCR assay (Applied Biosystems). The abundance of individual mRNA transcript in each sample was assayed three times and normalized to that of porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (as an internal control). The primers were designed with Primer Express software v.3.0 (Applied Biosystems). The sequences of the primers are listed in Table S2.

**In vitro deubiquitination assay**

Wild-type HA-tagged USP25 protein was purified from cells transfected with pCAGGS-HA-USP25 using a HA tagged Protein PURIFICATION KIT (MBL) according to the manufacturer’s protocol. As a negative control, a HA tagged Protein PURIFICATION KIT was also used to isolate proteins from empty-vector transfected cells. Polyubiquitin chains were purchased from Boston Biochem (K48-Ub, K63-Ub) (Catalog No. UC-230) and K63-Ub (Catalog No. UC-330). The purified products (2 μL) were incubated with 3.5 μL of K48-Ub, K63-Ub chains or K63-Ub, chains at 37°C in a 14.5 μL reaction mixture containing 25 mM NaCl, 100 μg/mL bovine serum albumin (BSA), and 2 mM dithiothreitol (DTT). A control reaction mixture was incubated under identical conditions with the exclusion of the enzyme. Reactions were terminated by addition of 5 × SDS-PAGE sample loading buffer (Beyotime, China) followed by heat treatment at 100°C for 10 min. Samples were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel and stained with Coomassie blue dye. Reaction mixtures were boiled with sample buffer and then proteins were separated by SDS-PAGE.

**Assay of deubiquitination activity in vivo**

HEK-293T cells cultured in 60-mm dishes were co-transfected with 1 μg of Flag-Ub, HA-K48-Ub, or HA-K63-Ub in addition to the appropriate amount of constructs containing USP25 or the corresponding mutants using Lipofectamine 2000. Where applicable, the empty pCAGGS-HA or pCAGGS-Myc vector was supplemented to keep the total amount of DNA transfected constant. After 30 h, cells were harvested by adding 200 μL 2 × lysis buffer A (LBA) (65 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 3% DL-dithiothreitol, and 40% glycerol) containing 20 mM N-ethylmaleimide (NEM) (Sigma-Aldrich) and 20 mM iodoacetamide (Sigma-Aldrich). Cell lysates were then analyzed for ubiquitin-conjugated proteins by Western blotting with anti-HA antibodies (1:1,000) (ABclonal Biotechnology) or anti-Flag antibodies (1:1,000) (Macgene, China). To confirm the expression levels of USP25 and the mutants, anti-HA antibodies were used to detect HA-tagged proteins, and anti-Myc antibodies (Beyotime, China) were used to detect Myc-tagged proteins. Beta-actin was detected with anti-beta-actin monoclonal antibodies (MAB) (Beyotime, China) to demonstrate equal protein sample loading.

**Co-immunoprecipitation and immunoblotting analysis**

For transient transfection experiments, HEK-293T cells were transfected for 28 h. The transfected cells were lysed in 200 μL of lysis buffer (4% SDS, 3% DTT, 0.065 mM Tris-HCl, pH6.8, 30% glycerin) supplemented with protease inhibitors (PMSF). Lysates were boiled at 100°C for 10 min before being separated by SDS-PAGE and then electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad), and analyzed by Western blotting with the indicated antibodies. Anti-USP25, anti-IRF3, anti-phospho-IRF3, anti-NF-κB p65, anti-phospho-NF-κB p65 and anti-ubiquitin antibodies were purchased from AbClonal Biotechnology. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

For co-immunoprecipitation analysis, Cells were washed with phosphate-buffered saline and lysed for 20 min at 4°C in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 10% glycerin, 0.1% SDS, and 2 mM Na2EDTA. Lysates were then cleared by centrifugation, and proteins were immunoprecipitated with affinity antibodies and protein A+G agarose beads (Beyotime, China) at 4°C. Immunoprecipitates were washed three times with 1 mL of lysis buffer. The precipitates were analyzed by standard immunoblot procedures.

**Statistical analysis**

All experiments were performed at least three times with reproducible results. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) without interaction terms followed by Dunnett’s for multiple comparisons. A P-value of <0.05 was considered statistically significant.

**Results**

**USP25 negatively regulates virus-induced activation of ISRE and the expression of ISGs**

As mentioned above, ISRE, as an IFN stimulation of promoter, plays a central role in the JAK-STAT signal transduction pathways, which leads to transcriptional induction of a wide range of downstream antiviral genes and the subsequent innate antiviral response. To identify potential USPs that might regulate the antiviral innate immune response, we screened a pool of 162 siRNAs targeting 54 human USPs genes for their abilities to regulate SEV-induced activation of the ISRE promoter in reporter assays in HEK-293T cells. HEK-293T cells were transfected with siRNA pools (three siRNAs per gene) as well as ISRE luciferase reporter plasmid. Twenty-four hours after transfection, cells were either uninfected or infected with SEV for 16 h before reporter assays were performed. As listed in Figure 1A, several siRNA pools potently potentiated SEV-induced activation of the ISRE promoter. Our further analysis focused on USP25, since a previous study demonstrated that USP11, which is the top-ranking candidate in this study, was constitutively associated with IκBα and attenuated IκBα degradation to negatively regulate TNFα-induced NFα-xB activation [34].

To validate the effect of USP25 knockdown on the SEV-induced ISRE reporter activity, we further assessed whether overexpression of USP25 also affects ISRE reporter activity. HEK-293T cells were transfected with ISRE reporter plasmid...
Figure 1

(A) A siRNA screen for USPs functions on SEV-induced activation of the ISRE promoter. HEK-293T cells were transfected with an ISRE luciferase reporter (0.1 μg) and control Renilla luciferase reporter (0.02 μg) vectors and NC (control siRNA) or specific siRNA pools (three siRNAs per gene) of members of USPs subclass of deubiquitinase for 24 h, and then infected or mock-infected with SEV for 16 h before luciferase assays were performed. (B) USP25 inhibited SEV-induced activation of ISRE. ISRE luciferase reporter (0.1 μg) and control Renilla luciferase reporter (0.02 μg) vectors were co-transfected into HEK-293T cells with either an empty vector (1 μg) or increasing amounts of USP25 (0.25, 0.5, or 1 μg) for 24 h. Cells were then either untreated or treated with SEV for 16 h before the relative luciferase activity was measured and normalized with the Renilla activity. Error bars indicate ± SD in three independent experiments. (C–G) USP25 significantly reduced the transcription of multiple ISGs. Empty vector (1 μg) or expression plasmid of USP25 (1 μg) were transfected into HEK-293T cells for 24 h and either untreated or treated with SEV for 16 h before real-time RT-PCR was performed.

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and increasing amounts of HA-USP25 expression plasmids. The results suggest that overexpression of USP25 strongly inhibited SEV-induced activation of ISRE promoter in a dose-dependent manner (Figure 1B).

We next asked whether overexpression of USP25 affects the expression of ISGs, whose promoters contain the ISRE binding domain. To this end, HEK-293T cells were treated with SEV, and ISG expression was assessed after 16 h. We specifically measured the effect of overexpression of USP25 on mRNA levels of five ISGs (ISG15, ISG20, ISG54, ISG56 and IP-10). Real-time RT-PCR analysis suggested that the mRNA levels of ISG15 (Figure 1C), ISG20 (Figure 1D), ISG54 (Figure 1E), ISG56 (Figure 1F) and IP-10 (Figure 1G) were markedly reduced after stimulation in HEK-293T cells. These results suggest that USP25 indeed negatively regulates the antiviral innate immune response.

**USP25 Impacts On the Antiviral Innate Immune Response by Reduction of IFN-β Expression**

It is well known that the IFN system constitutes the primary defense against viral infections. Therefore, we investigated the effect of overexpression of USP25 on virus-induced type I IFN signaling. We used an IFN-β-dependent luciferase reporter assay to assess its abilities to modulate SEV-induced IFN-β promoter in HEK-293T cells. The results showed that USP25 significantly reduced SEV-induced IFN-β luciferase reporter activity (Figure 2A). Consistent with these observations, quantitative reverse transcription (RT)-PCR showed that overexpression of USP25 inhibited SEV-induced expression of IFN-β (Figure 2B). In addition, poly (I:C)-induced production of IFN-β was significantly attenuated by the expression of USP25 (Figure 2C). We further investigated the effect of USP25 knockdown on virus-triggered IFN signaling. And we first tested the effect of individual siRNAs targeting USP25 by quantitative reverse transcription (RT)-PCR and immunoblotting. The results showed that expression of cell-endogenous USP25 were reduced both at the level of mRNA and protein (Figure 2D and E). Reporter assays then indicated that knockdown of USP25 markedly potentiated SEV-induced activation of the IFN-β promoter (Figure 2F). Collectively, these findings suggest that USP25 inhibits RIG-I/MDA5-dependent type I IFN signaling.

**USP25 inhibits SEV-induced type I IFN signaling by disrupting activation of IRF3 and NF-κB**

To better understand the effects of USP25 in SEV-induced type I IFN signaling, we assessed whether expression of USP25 disrupts SEV-induced activation of IRF3 and NF-κB, the two important transcriptional factors in type I IFN signaling. We observed that overexpression of USP25 significantly inhibited SEV-induced activation of IRF3 and NF-κB (Figure 3A and B). Previous study indicated that porcine reproductive and respiratory syndrome virus (PRRSV) NSP1β could inhibited both IFN regulatory factor 3 (IRF3)- and NF-κB-dependent gene induction by dsRNA and Sendai virus [35]. Therefore, we used NSP1β as a positive control in Figure 3A and B. We further examined whether USP25 affects virus-induced phosphorylation of IRF3 and p65. The results showed that overexpression of USP25 strongly potentiated SEV-induced phosphorylation of IRF3 and p65 (Figure 3C and D). Taken together, our results suggest that USP25 negatively regulates IFN-β expression by inhibiting SEV-induced activation of IRF3 and NF-κB.

**USP25 processes K48-linked and K63-linked polyubiquitin in vitro and in vivo**

USPs are cysteine proteases that vary greatly in size and structural complexity. Thus, it is unlikely that all predicted USPs are truly specific for Ub [23]. To identify if USP25 has DUB activity, a DNA construct expressing USP25 was transiently transfected into HEK-293T cells and the recombinant USP25 was purified from cell lysates using a HA tagged Protein PURIFICATION KIT (MBL) (Figure 4A). When incubated with K48- and K63-linked polyubiquitin chains in vitro, the purified USP25 was highly effective in cleaving both substrates into monomers (Figure 4B and C).

To further determine whether USP25 has DUB activity in a cell-based assay, HEK-293T cells were transfected with either an empty vector or increasing amounts of plasmid DNA encoding USP25 along with a Flag-tagged ubiquitin vector (Flag-Ub), and the effect of USP25 on all ubiquitinated cellular proteins was assessed via Western blotting with an anti-Flag antibody. As shown in Figure 5A, overexpression of USP25 resulted in a dose-dependent reduction in the levels of ubiquitinated cellular proteins compared with those levels observed in the control vector-transfected cells. To further identify which Ub linkage type is targeted by USP25 in vivo, HEK-293T cells were transfected with HA-K48-Ub or HA-K63-Ub in lieu of HA-Ub. These constructs allow solely for the formation of K48- and K63-linked polyubiquitin chains, respectively. As shown in Figure 5B and C, transfection with USP25 resulted in an obvious reduction in the extent of conjugation of K48- and K63-linked polyubiquitin. These observations, together with the results from the deubiquitination assays in vitro, clearly demonstrated that USP25 possesses DUB activity and affects both K48-linked and K63-linked ubiquitination.

**Identification of USP25 DUB activity sites**

Based on the structures of their catalytic domains, the human DUBs have been classified into five subfamilies, most of which exhibit a high degree of homology mainly in two regions known as Cys and His boxes (C and H boxes, respectively) that surround the catalytic Cys and His residues [23,36]. We chose six USPs whose DUB activity had been reported [34,37–41]. Sequence alignment showed that Cys178 and His607 of USP25 are highly conserved among all seven human DUBs and are critical to USP25's catalytic activity.

To further determine the catalytic residues of USP25, we used a cell-based assay, HEK-293T cells were transfected with either an empty vector or increasing amounts of plasmid DNA encoding USP25 along with a Flag-tagged ubiquitin vector (Flag-Ub), and the effect of USP25 on all ubiquitinated cellular proteins was assessed via Western blotting with an anti-Flag antibody. As shown in Figure 5A, overexpression of USP25 resulted in a dose-dependent reduction in the levels of ubiquitinated cellular proteins compared with those levels observed in the control vector-transfected cells. To further identify which Ub linkage type is targeted by USP25 in vivo, HEK-293T cells were transfected with HA-K48-Ub or HA-K63-Ub in lieu of HA-Ub. These constructs allow solely for the formation of K48- and K63-linked polyubiquitin chains, respectively. As shown in Figure 5B and C, transfection with USP25 resulted in an obvious reduction in the extent of conjugation of K48- and K63-linked polyubiquitin. These observations, together with the results from the deubiquitination assays in vitro, clearly demonstrated that USP25 possesses DUB activity and affects both K48-linked and K63-linked ubiquitination.

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Figure 2. USP25 significantly inhibits virus-induced type I IFN signaling. (A) USP25 inhibited SEV-induced activation of the IFN-β promoter in a dose-dependent manner. IFN-β luciferase reporter (0.1 μg) and control Renilla luciferase reporter (0.02 μg) vectors were co-transfected into HEK-293T cells with an empty vector (1 μg) or increasing amounts of USP25 (0.25, 0.5, or 1 μg) for 24 h. Cells were then either untreated or treated with SEV for 16 h before the relative luciferase activity was measured and normalized with the Renilla activity. Error bars indicate ± SD in three independent experiments. (B) USP25 significantly inhibited the transcription of IFN-β. Empty vector (1 μg) or expression plasmid of USP25 (1 μg) were transfected into HEK-293T cells for 24 h and either untreated or treated with SEV for 16 h before real-time RT-PCR was performed. (C) USP25 inhibited poly (I:C)-induced activation of the IFN-β promoter in a dose-dependent manner. IFN-β luciferase reporter (0.1 μg) and control Renilla luciferase reporter (0.02 μg) vectors were co-transfected into HEK-293T cells with either an empty vector (1 μg) or increasing amounts of USP25 (0.25, 0.5, or 1 μg) for 24 h. Cells were then either untreated or treated with 1 μg of poly (I:C) for 16 h before the relative luciferase activity was measured and normalized with the Renilla activity. Error bars indicate ± SD in three independent experiments. (D, E) Effects of USP25 siRNA on endogenous USP25. HEK-293T cells were transfected with the indicated siRNA (20 nm each) for 24 h, and cell lysates were analyzed by quantitative reverse transcription (RT)-PCR (D) or immunoblots with antibodies against USP25 and β-actin (E). (F) Effects of USP25 siRNA on SEV-induced activation of the IFN-β promoter. HEK-293T cells were transfected with an IFN-β luciferase reporter (0.1 μg) and control Renilla luciferase reporter (0.02 μg) vectors and the indicated siRNA plasmids (20 nm each) for 24 h and then infected with SEV or uninfected for 16 h before luciferase assays were performed. Error bars indicate ± SD in three independent experiments. *P < 0.05 compared with cells transfected with Control followed by SEV infection. Significant differences between groups were determined by one-way ANOVA followed by Dunnett’s multiple comparisons test.

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Mutation of the catalytic residues results in significant loss of ability of USP25-mediated IFN inhibition

To examine whether the inhibitory effects of USP25 on SEV-induced type I IFN signaling is due to its deubiquitinase activity, wild-type USP25 (USP25-WT) and its mutants (C178A and H607A) lacking DUB activity were co-transfected with the promoter luciferase reporter plasmid of IFN-β, IRF3, NF-κB and ISRE, and the luciferase activity was detected. The results showed that USP25-WT remarkably inhibited SEV-induced activation of IFN-β (Figure 6A), IRF3 (Figure 6B), NF-κB (Figure 6C) and ISRE (Figure 6D) in a dose-dependent manner.
manner. However, the catalytic mutants (C178A and H607A) devoid of DUB activity lost the ability of USP25 WT-mediated IFN inhibition to some degree, indicating that DUB activity is involved in USP25 inhibition of type I IFN induction.

USP25 deubiquitinates RIG-I, TRAF2 and TRAF6

To further determine the levels at which USP25 negatively regulates type I IFN signaling, HEK-293T cells were transfected with DNA constructs encoding RIG-I, TRAF2, or TRAF6, together with IFN-β-Luc. As shown in Figure 7A, overexpression of RIG-I, TRAF2, or TRAF6 significantly activated IFN-β promoter compared with cells transfected with an empty vector control. However, such effects were all substantially reduced in the presence of USP25.

Since the DUB activity of USP25 was involved in virus-induced type I IFN signaling, we further investigated whether the IFN antagonist function of USP25 is associated with the deubiquitination of RIG-I, TRAF2, and TRAF6, which are essential signaling components in the type I IFN pathway activated by virus infection. We observed that overexpression of USP25 significantly inhibited ubiquitination of RIG-I (Figure 7A).

Figure 4. Processing of K48- and K63-linked polyubiquitin chains by USP25 in vitro. (A) Analysis for purified HA-tagged USP25 conjugated proteins. The protein was obtained from USP25-transfected or mock-transfected HEK-293T cells using a HA tagged Protein PURIFICATION KIT (MBL) and analyzed for HA-tagged USP25-conjugated proteins by western blotting (WB) with an anti-HA antibody. (B) In vitro K48-linked polyubiquitin deconjugation assay. K48-linked polyubiquitin was incubated with the protein obtained from mock-transfected (lane 2) or USP25-transfected (lane 3) HEK-293T cells at 37°C for 1 h before being analyzed by SDS-PAGE. Lane 1, uncleaved K48-linked polyubiquitin chain (K48-Ub2–7). M, molecular mass markers, including 170-, 130-, 100-, 70-, 55-, 40-, 35-, 25-, 15-, and 10-kDa bands. (C) In vitro K63-linked polyubiquitin deconjugation assay. The experiment was performed similarly as in B, except that the K63-linked polyubiquitin chain (K63-Ub2–7) was used.

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Figure 5. USP25 has a dose-dependent deubiquitinating activity *in vivo*. (A) HEK-293T cells grown in 60-mm dishes were transfected with Flag-tagged Ub expression plasmids (1 μg), along with increasing quantities (0.25, 0.5, 1, or 2 μg) of plasmid encoding USP25 using Lipofectamine 2000. Cell lysates were prepared at 30 h post-transfection and analyzed for Ub-conjugated proteins by Western blotting with an anti-Flag antibody. Western blotting with anti-HA antibodies shows expression of USP25, and Western blotting for beta-actin served as a protein loading control. (B, C) USP25 effectively cleaved both K48 and K63 Ub linkages *in vivo*. The experiment was performed similarly to that described for panel A except that HA-K48-Ub or HA-K63-Ub was used in lieu of Flag-Ub and different expression plasmids of USP25 were used. (D) Mapping of the putative sites are associated with the DUB activity of USP25. Black boxes indicate conserved residues tested in this study. The sequences were derived from GenBank entries with the following accession numbers: USP2, NM_004205; USP4, NM_003363; USP11, NM_004651; USP17, NM_201402 and XM_352721; USP20, NM_001110303; USP21, NM_001014443; and USP25, NM_013396. (E) Cysteine 178 (C178) and histidine 607 (H607) are deubiquitination active sites of USP25. Expression vectors encoding pcDNA3.1-Flag-Ub and control vectors or expression vectors encoding HA-USP25-WT, -C178A and -H607A were co-transfected into HEK-293T cells. USP25-WT and deletion mutant proteins in cell lysates were immunoprecipitated with anti-HA antibodies under denaturing conditions and immunoblotted with anti-Flag antibodies to detect the presence of Ub-conjugated proteins. Western blotting for beta-actin served as a protein loading control.

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7B), TRAF2 (Figure 7D), and TRAF6 (Figure 7E). In contrast, the USP25 mutants (C178A and H607A) lacking DUB activity had no such effects. However, overexpression of USP25 did not block ubiquitination of IPS-1 (Figure 7C). Taken together, our results confirmed that USP25 acts as a deubiquitinase of RIG-I, TRAF2 and TRAF6 to inhibit virus-induced type I IFN signaling.

Discussion

The production of type I IFNs and induction of interferon-inducible genes after virus infection are central to antiviral innate immune responses. Ubiquitination and deubiquitination have emerged as critical post-translational regulatory mechanisms for activation or attenuation of the virus-triggered IFN response pathway. Previous studies have demonstrated a critical role for the members of DUBs in antiviral innate immune responses. For example, several deubiquitinating enzymes, including A20, Cezanne, CYLD, USP15, and USP31, have been suggested to be involved in the downregulation of TNF-α induced NF-κB activation [27,42–45]. In recent years, the USPs subclass of DUBs has been deemed to represent the bulk of the deubiquitinating enzymes encoded in the human genome, and their distribution and functional diversity in eukaryotic tissues and organs have received extensive attention. However, the roles of USPs in the regulation of type I IFN
Figure 7. USP25 deubiquitinates RIG-I, TRAF2 and TRAF6. (A) USP25 inhibited RIG-I-, IPS-1-, TRAF2-, and TRAF6-mediated activation of the IFN-β promoter. HEK-293T cells were transfected with an IFN-β promoter reporter (0.1 µg) and either an empty vector or the indicated plasmids encoding the RIG-I, IPS-1, TRAF2, and TRAF6 expression vector (1 µg each) for 30 h before luciferase assays were performed. *p < 0.05 for all pairwise comparisons by one-way ANOVA followed by Dunnett’s multiple comparisons test. (B–E) HEK-293T cells were co-transfected with the indicated plasmids encoding the RIG-I (B), IPS-1 (C), TRAF2 (D), or TRAF6 (E) expression vector (4 µg) and HA-USP25WT/ HA-USP25C178A/ HA-USP25H607A (4 µg) using Lipofectamine 2000. The immunoprecipitates (IP) were analyzed by immunoblots (IB) with anti-ubiquitin (top panels) and anti-Flag (middle panels). The levels of the transfected USP25/mutants were detected by immunoblots with anti-HA (bottom panels). The input tagged proteins were verified with the indicated antibodies.

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production have not been studied in great detail. In this study, we identified USP25 as a major deubiquitinase of RIG-I, TRAF2, and TRAF6 in SEV-induced type I IFN induction.

Of the DUBs family, USPs have raised special interest because of the multiple family members described in different eukaryotic organisms. It is thought that not all USPs that contain well-conserved motifs have DUB activity. Recently, Quesada et al. reported that USP40, USP47, and USP48 show no activity, despite possessing all the catalytic residues proposed as being important for the catalytic activity of these enzymes [46]. However, DUB activity had been demonstrated for many USPs. For example, Junichiro et al. demonstrated that USP21 constitutively deubiquitinates RIP1 in vitro and in vivo [40]; and a previous study has reported that USP20 deubiquitinates TRAF6 and Tax in vivo [40]. Here, we found that USP25 contains conserved Cys178 and His607 residues, which are important for the catalytic activity of USPs, suggesting that USP25 may have deubiquitinating activity. In subsequent experiments, we observed that USP25 did indeed appear to have DUB activity both in vitro and in vivo. We also revealed that USP25 could act on both K48- and K63-linked Ub polymers. In addition, we also found that Cys178 and His607 residues are responsible for the DUB activity of USP25.

By using a siRNA screen, we found that knockdowns of several USPs potently potentiated SEV-induced activation of the ISRE promoter, some of which had been reported as being associated with the NF-kB signaling pathway. For example, USP11 negatively regulates TNFα-induced NF-κB activation associated with IkBα and attenuates IkBα degradation [34]; USP20 deubiquitinates TRAF6 and suppresses interleukin 1β (IL-1β)- and Tax-induced NF-κB activation [40]; Katrin et al. showed that USP15 regulates IkBα/NF-κB by deubiquitinylation IkBα[44]; and USP31 inhibits TNFα, CD40, TRAF2, TRAF6 and IKKβ-mediated NF-κB activation [45].

It is well known that the IFN-β promoter contains conserved enhancer elements recognized by NF-κB that lead to induction of IFN-β, which then activates ISRE. Thus, these USPs (USP11, USP20, USP15, and USP31) are very likely to inhibit virus-induced ISRE reporter activity. Consistent with this supposition, we found that knockdowns of these USPs strengthened SEV-induced ISRE reporter activity. In this study, we focus on USP25, whose knockdown significantly potentiated SEV-induced activation of the ISRE promoter in the siRNA screen. However, this screening serves only as an initial step to identify the genes. We further found that overexpression of USP25 efficiently reduced SEV-induced IFN-β induction.

Ubiquitination and deubiquitination are critical players in modulating the antiviral innate immune response. Several ubiquitin ligase enzymes have been found to regulate these processes [47,48]. For example, ubiquitination of RIG-I by the E3 ubiquitin ligase TRIM25, which contains a RING finger domain, is necessary and sufficient to activate IPS-1, which triggers the downstream signaling cascade to produce type I IFN [49]. Virus-triggered ubiquitination of TRAF2/6 by cIAP1/2 is essential for induction of IFN-β and the cellular antiviral response [50,51]. However, certain cellular USPs are known to modulate the antiviral innate immune response. For example, USP17 and USP20 were found to target RIG-I and TRAF6 respectively, thereby functioning as novel regulators of antiviral innate immune responses [39,40]. In the present study, we are the first to exped to that USP25 inhibited RIG-I- , IPS-1- , TRAF2-, and TRAF6-mediated activation of the IFN-β promoter. In addition, wild-type USP25 significantly inhibits ubiquitination of RIG-I, TRAF2, and TRAF6, which is essential for activation of type I IFN signaling. However, both catalytically inactive mutants that are defective for DUB activity lost the capability of reducing ubiquitinated RIG-I, TRAF2, and TRAF6. In addition, the catalytic USP25 mutants (C178A and H607A) devoid of DUB activity significantly lost the ability of USP25 WT-mediated IFN inhibition. Taken together, these results indicate that the DUB activity of USP25 is involved in the inhibition of type I IFN induction. However, inhibition of DUB activity by mutagenesis means did not completely abrogate the ability of USP25 to block viral activation of the type I IFN signaling pathway. We hypothesize that mutation of the catalytic residues does not abolish the ability to combine with ubiquitin of target proteins; and that it is this kind of combination that may reduce the ability of proteins to transmit signals without cleaving the ubiquitin chains. Indeed, co-immunoprecipitation investigation showed that USP25 interacted with RIG-I and TRAF6 (see Figure S1). Therefore, we hypothesize that USP25 may affect RIG-I-mediated signal transmission by interacting with RIG-I and TRAF6 to reduce type I interferon induction. Further investigation will be required to identify USP25 mutants that disrupt the interaction of USP25 and RIG-I/TRAF6 but do not affect DUB activity; and to test whether these domains of USP25 interacting with RIG-I and TRAF6 also contribute to the inhibition of IFN induction by USP25.

In recent years, USP25 has been extensively studied and some novel functions have been revealed. Previous studies have shown that the DUB activity of USP25m, a muscle isoform of USP25, did not strictly depend on the ubiquitin-binding domains (UBDs), but required a coiled-coil stretch between amino acids 679 to 769 [52]. Future investigation will be required to test whether these domains of USP25 are also involved in USP25-mediated IFN inhibition. In addition, USP25 interacts with the ubiquitin ligase HRD1 and rescues several endoplasmic reticulum-associated degradation (ERAD) substrates from degradation by the proteasome [53]. Also, USP25 can be a functional deubiquitinase of TRAF5 and TRAF6 in the regulation of IL-17-mediated signaling and inflammation [54]. Recently Zhong et al. reported that USP25 was a critical modulator of TLR4-mediated, but not TLR3-mediated, signaling. They mainly focus on the regulation of USP25 deubiquitination in TLR4 signaling in terms of the production of proinflammatory cytokines and type I IFNs [55]. However, our studies explain that USP25 negatively modulates RIG-I/MDA5-dependent type I IFN signaling. These have showed that USP25 has a wide range of biological activity and differentially regulates innate response.

In our study, we observed USP25 to act as a DUB that cleaved ubiquitin chains from RIG-I, TRAF2, and TRAF6, thereby inhibiting SEV-induced type I IFN signaling. In conclusion, our studies suggest that USP25 serves as another
level of critical regulatory control to maintain a delicate balance in virus-induced type I IFN signaling.

Supporting Information

Figure S1. USP25 interacts with RIG-I and TRAF6. (A, B) HEK-293T cells grown in 100-mm dishes were co-transfected with the indicated plasmids encoding the RIG-I (A) or TRAF6 (B) expression vector (4 μg) and HA-USP25WT/HA-USP25C178A/HA-USP25H607A (4 μg) using Lipofectamine 2000. Immunoprecipitation (IP) was analyzed by immunoblots (IB) with anti-HA (top panels) and anti-Flag (middle panels). The input tagged proteins were verified with the indicated antibodies.

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