Antiviral Research 100 (2013) 546–554

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

PIAS1 negatively modulates virus triggered type I IFN signaling by blocking the DNA binding activity of IRF3

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ARTICLE INFO

Article history:
Received 19 August 2012
Revised 30 August 2013
Accepted 2 September 2013
Available online 12 September 2013

Keywords:
PIAS1
Interferon
IRF3
SUMOylation
Transcription

ABSTRACT

During viral infection, production of proinflammatory cytokines including type I interferons (IFNs) is under stringent control to avoid detrimental overreaction. The protein inhibitor of activated STAT (PIAS) family proteins have been recognized as anti-inflammatory molecules by restraining type I IFN induced amplifying signaling. Here we identified PIAS1 as an important negative regulator of virus-triggered type I IFN signaling. Overexpression of PIAS1 repressed virus-or RIG-I like receptor stimulated type I IFN transcription, whereas knockdown of PIAS1 expression augmented virus-induced production of type I IFNs. PIAS1 with a mutation in the SAP domain retained the inhibitory function in virus-induced IFN transcription, but abolished the inhibition in IFN-stimulated signaling. SUMO E3 ligase activity dead mutant PIAS1/C350S still had the comparable inhibitory function with WT PIAS1. Further study indicated that PIAS1 interacted with IRF3 and inhibited the DNA binding activity of IRF3. The C-terminal region of PIAS1 around a cluster of acidic amino acids is critical for the interaction with IRF3 and the inhibitory functions of PIAS1. Therefore, these results unveil PIAS1 functions both at the virus-induced early signaling stage and IFN stimulated amplifying stage with distinct mechanisms. PIAS1 is important in maintaining proper amounts of type I IFNs and restrains its magnitude when the antiviral response intensifies.

1. Introduction

Innate immunity constitutes the first line of defense against pathogens in the host. Infection of the RNA viruses is recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I like receptors (RLRs) and triggers a series of signaling cascades that lead to induction of proinflammatory cytokines including type I IFNs (Akira et al., 2006; Andrejeva et al., 2004; Kato et al., 2006; Kawai and Akira, 2006; Takeda and Akira, 2005; Yoneyama and Fujita, 2009; Yoneyama et al., 2004).These PRRs may have different recognition preference and trigger distinct or similar signaling pathway. TLR3 recognizes dsRNA and transmits signals via TRIF-dependent manner (Yamamoto et al., 2003). The RLRs, including RIG-I and MDA-5 sense 5′-triphosphate RNA viruses and signal through VISA (also known as Cardif, MAVS) dependent manner (Xu et al., 2005; Yoneyama et al., 2004). To ensure efficient elimination of infection and minimize harmful excess inflammatory responses, TLR- and RLR- mediated production of proinflammatory cytokines are finely modulated both positively and negatively at multiple steps of the signaling cascades. Many proteins have been reported to act as negative regulators in this process (Komuro et al., 2008; O'Neill, 2008; Wang et al., 2009a).

PIAS family comprises four members, PIAS1, PIASx (PIAS2), PIAS3 and PIASy (PIAS4) (Shuai, 1999, 2000; Shuai and Liu, 2003). They were initially identified as negative regulators of activated STAT dependent transcription (Chung et al., 1997; Liu et al., 1998). These members exhibit distinct or redundant roles in multiple signaling pathways. They all contain a conserved RING-finger zinc binding domain (RLD) and this domain confers them SUMO-E3-ligase activity (Jackson, 2001; Schmidt and Muller, 2003). Many proteins have been reported as the substrates for SUMOylation, most of which are transcription factors. In addition to STATs, PIAS proteins also regulate a large number of transcription factors involved in cell cycle control, immune responses and development (Liu et al., 1998; Shuai and Liu, 2005). PIAS proteins repress transcription through four major mechanisms: blocking the DNA binding activity of a transcription factor; recruiting transcriptional co-regulators; promoting the SUMOylation of a transcription factor and sequestering transcription factors in certain subnuclear structures that are enriched for corepressor complexes (Shuai and Liu, 2005).

Our previous study demonstrates that PIASy negatively regulates TLR3 dependent virus signaling (Zhang et al., 2004). This was also demonstrated by other group (Kubota et al., 2011). Like other PIAS family members, PIAS1 is also an anti-inflammatory

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http://dx.doi.org/10.1016/j.antiviral.2013.09.001

molecule (Liu and Shuai, 2008; Liu et al., 2007). PIAS1 can regulate the transcriptional activity of STAT1 and other transcription factors including p53, androgen receptor, p65 and smads (Liang et al., 2004; Liu et al., 1998, 2005; Nishida and Yasuda, 2002; Schmidt and Muller, 2002). PIAS1 can directly block the binding of STAT1 or p65 with the chromosome (Liu et al., 1998, 2005). Compared to the unobvious phenotype in *Piasy* deficient mice, *Pias1* deficient mice display increased transcription of a subset of STAT1 and NF-κB induced genes, resistance to viral and bacterial infections, increased serum levels of proinflammatory cytokines (Liu et al., 2004; Roth et al., 2004; Wong et al., 2004). These results imply a crucial role for PIAS1 in innate immune responses and indicate that PIAS1 may have more critical functions in gene regulation than PIASy. Therefore, it raised our interests to investigate whether PIAS1 is involved in virus induced type I IFN signaling. In the present study, We demonstrate that PIAS1 is an important negative regulator of virus induced type I IFN signaling.

2. Materials and methods

2.1. Reagents

Mouse monoclonal antibodies against Flag (Sigma–Aldrich, St. Louis, MO) or hemagglutinin epitope (HA) (Cali-Bio, USA, or Tianjin Sungene Biotech, China), polyclonal antibody against GAPDH (Proteintech Group Inc., Chicago, IL), PIAS1 antibody (Origene, Rockville, MD), IRF-3 (Santa Cruz), phospho-IRF3 antibody (Merck Millipore, Billerica, MA), poly(I:C) (InvivoGen, San Diego, CA) and transfection reagents Lipofectamine 2000 (Life Technologies, Carlsbad, CA), jetPrime (Polyplus-transfection, IIIkirch, France) and VigoFect (Vigorous, Beijing, China) were purchased from the indicated manufactures.

2.2. Cell cultures

Human embryonic kidney cell line HEK293, HEK293T, and cervical carcinoma cell line HeLa, were grown in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% neonatal calf serum supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and sodium bicarbonate (2.2 g/L).

2.3. Plasmid constructs

Mammalian expression plasmids for human Flag or HA-tagged PIAS1 were constructed by PCR amplification of PIAS1 open reading frame from myc-tagged PIAS1 vector by standard molecular biology techniques. Mammalian expression plasmids for PIAS1/mSAP, PIAS1/C350S, PIAS1/ΔSIM, PIAS1/1-520, PIAS1/1-460, PIAS1/1-400, PIAS1/1-311 (PIAS1 splicing), PIAS1/S90D and PIAS1/S90A mutants were constructed by site-directed mutagenesis. All these vectors were verified by sequencing.

2.4. RNA interference

Four shRNA plasmids against PIAS1 were provided by GenePharma (Shanghai, China). ShRNA against PIAS1 and the non-silencing control were transfected into HEK293 cells using the jetPRIME reagent according to the manufacturer's protocol. PCR amplifications of PIAS1 were performed using the following primers: PIAS1-F:5′-TCTTACAATGGAGTCGATGGAT-3′ and PIAS1-R:5′-GCTGCTGTTACTGCCACTACTG-3′. PCR products were resolved on 1% agarose gels. The cell lysates were also blotted by anti-PIAS1 antibody (Origene) at 1:1000 dilution.

2.5. Cell transfection and luciferase reporter assays

Cells (1.2×10^5) were seeded on 24-well plates and transfected the next day using VigoFect with 100 ng ISRE luciferase reporter or IFN-β reporter and 1 ng pRL-SV40 plasmid. In the same experiment, when necessary, empty control plasmid was added to ensure that each transfection received the same amount of total DNA. Then, twenty-four hours after transfection, cells were stimulated with SeV or transfected with poly (I:C) (4 μg/ml) using Lipofectamine 2000 for twenty hours and luciferase activities were measured by Dual Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized based on Renilla luciferase activity. All reporter assays were repeated at least three times. Data shown are average values ±SD from one representative experiment. Sendai virus infection was performed as described (Zhang et al., 2004).

2.6. Reverse transcriptase-PCR and real-time PCR

Total RNA was isolated by TRIzol reagent (Invitrogen). RNA was reversely transcribed using the Reverse Transcription System (Promega) according to the manufacture's instruction. Quantitative real-time polymerase chain reaction (PCR) was performed using the Bio-Rad Real-Time PCR system with Power SYBR Green PCR master mix (Bio-Rad). Each reaction was performed in triplicate, and negative controls were included in each experiment. The amounts of *IFNB1*, *ISG56*, *IL8* mRNA were amplified using the following primers: *IFNB1*-F:5′-ATTGCCTCAAGGACAGGATG-3′ and *IFNB1*-R:5'-GGCCTTCAGGTAATGCAGAA-3′; *ISG56*-F:5′-GCCATTTTCTTTGCTTCCCCTA-3′ and *ISG56*-R:5′-TGCCCTTTTGTAGCCTCCTTG-3′; *IL8*-F:5′-AGGTGCAGTTTTGCCAAGGA-3′ and *IL8*-R:5′-TTTCTGTGTTGGCGCAGTGT-3′.

2.7. Immunoprecipitation and western blot

HEK293T cells were plated at 3.5×10^6 cells per 10 cm dish and transiently transfected with 10 μg of HA-PIAS1 or PIAS1 mutants accompanied with 10 μg Flag-IRF3 using Vigofect according to the manufacturer's recommendations. Then cells were lysed in lysis buffer containing proteinase inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride. Cell lysates were incubated with 1 μg/ml anti-HA antibody or anti-Flag or control immunoglobulin (IgG) overnight. The immune complex was precipitated using protein-A Sepharose (GE Healthcare) and resolved by SDS–PAGE. The blot was then probed with anti-Flag or anti-HA antibody. IRDye-700 conjugated anti-IgG was used as a secondary antibody, and proteins were identified using the Odyssey Imaging System.

2.8. Immunofluorescence analysis

HEK293 cells (9×10^4) were seeded on 6-well plates and transfected with HA-PIAS1 or empty vector (1 μg each) the next day. After twenty-four hours, cells were infected with Sendai virus for six hours and fixed with 4% paraformaldehyde. After three washes, cells were permeabilized with 0.1% Triton X-100 for 5 min and incubated at 4 °C with a blocking solution (PBS containing 5% BSA) for 30 min. The primary antibodies were added into the blocking solution at a 1:100 dilution, and cells were incubated overnight at 4 °C. After three washes, cells were then incubated with secondary antibodies for one hour at 4 °C and counterstained with 2 μg/ml Hoechst for 15 min. Stained cells were viewed on Leica SP5 spectral scanning confocal microscope.

2.9. Chromatin immunoprecipitation (ChIP)

Approximately 5×10^7 HEK293T cells were used for each ChIP assay. It was performed by the ChIP assay kit (Millipore, Billerica, MA) according to the manufacturer's instructions. HEK293T cells were either uninfected or infected with Sendai virus for 8 h. Cell extracted were prepared, and chromatin were sheared by sonication (five 10 s pulses at 30% of the maximum strength). After sonication, protein–DNA complexes were immunoprecipitated from nuclear extracts by using rabbit polyclonal IRF-3 antibody (Santa Cruz) or normal rabbit IgG or anti-polymerase II or mouse IgG, followed by capture on protein A/G Sepharose beads. After IP and elution from the resin, the DNA was purified using the Qiaquick PCR Purification kit (Qiagen). Then the bound DNA was amplified by real-time PCR. The results are expressed as a signal ratio, which represents the signal to background (IgG) ratio.

The sequences of the amplification of *IFNB* promoter are: IFNβ-F:5′-GAATCCACGGATACAGAACCT-3′; IFNβ-R:5′-TTGACAACACGAACAGTGTCG-3′.

3. Results

3.1. Identification of PIAS1 as a negative regulator of virus induced IFN signaling

To investigate whether PIAS1 is involved in the regulation of virus-induced IFN signaling, mock control or PIAS1 expressing vector was overexpressed in HEK293T cells. As shown in Fig. 1A & B, overexpression of PIAS1 inhibited SeV-induced activation of both ISRE (an interferon stimulated response element) and IFN-β promoter in a dose dependent manner in HEK293T cells. To further support these results, the amount of *IFNB1*, *ISG56*, *IL8* mRNA was measured at various time points by quantitative real-time-reverse transcription PCR (qRT-PCR) during the twenty-four hours of infection by Sendai virus. Consistently, PIAS1 markedly inhibited SeV-induced transcription of endogenous *IFNB1* gene and the downstream gene *ISG56* and *IL8* (Fig. 1C). In addition, activation of the ISRE and IFN-β promoter primed with the synthetic RNA duplex poly (I:C) was also inhibited by overexpression of PIAS1 (Supplementary Fig. S1A). In accordance with the reporter assays, the transcription of endogenous *IFNB1* gene and the downstream *ISG56* and *IL8* induced by poly (I:C) was significantly inhibited as well (Supplementary Fig. S1B). The inhibitory effects of PIAS1 on virus-triggered activation of the IFN-β promoter and ISRE are not cell-type specific, similar results were observed in Hela cell line (Supplementary Fig. S2).Taken together, PIAS1 is a negative regulator of virus induced IFN signaling.

3.2. Knockdown of PIAS1 potentiates virus-triggered induction of IFN-β

We next investigated the function of endogenous PIAS1 in SeV-triggered type I IFN production. We screened four shRNA plasmids for PIAS1, and all these RNAi plasmids could reduce the expression of transfected and endogenous PIAS1 in HEK293 cells (Fig. 2A). The knockdown of PIAS1 by shRNA potentiated SeV-triggered transcription of endogenous *IFNB1*, *ISG56* and *IL8* in HEK293 cells (Fig. 2B). We also knockdowned PIAS1 by siRNA oligos against PIAS1 and obtained similar results (data not shown). These results together confirmed that PIAS1 plays a specific role in virus-triggered type I IFN signaling.

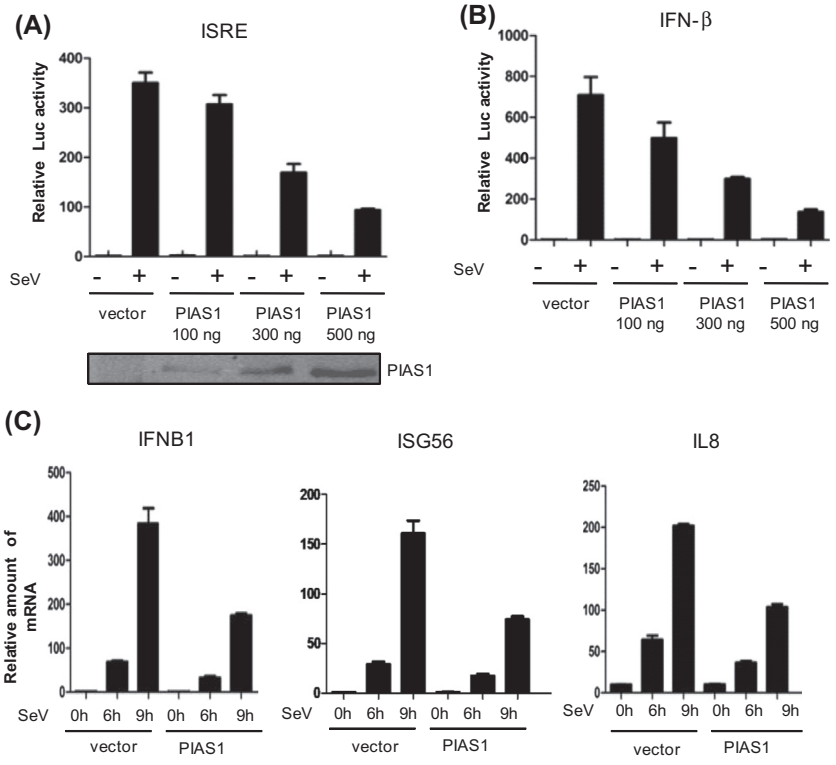

Fig. 1. PIAS1 inhibits virus-induced type I IFN signaling. (A) and (B) Overexpression of PIAS1 inhibited SeV-induced ISRE and IFN-β promoter activation in a dose-dependent manner. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with 100 ng ISRE luciferase reporter or IFN-β reporter and 1 ng pRL-SV40 plasmid, plus the indicated amount of PIAS1 plasmids. Twenty-four hours after transfection, cells were infected with SeV or left uninfected for twenty hours before luciferase assays were performed. (C) Overexpression of PIAS1 inhibited Sev-induced transcription of *IFNB1*, *ISG56* and *IL8*. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control or PIAS1 expressing vector (1 μg each). Twenty-four hours later, cells were left uninfected or infected with SeV. Cells were collected at the indicated time-point. Then real-time PCR analysis of the expression of *IFNB1*, *ISG56* and *IL8* was performed.

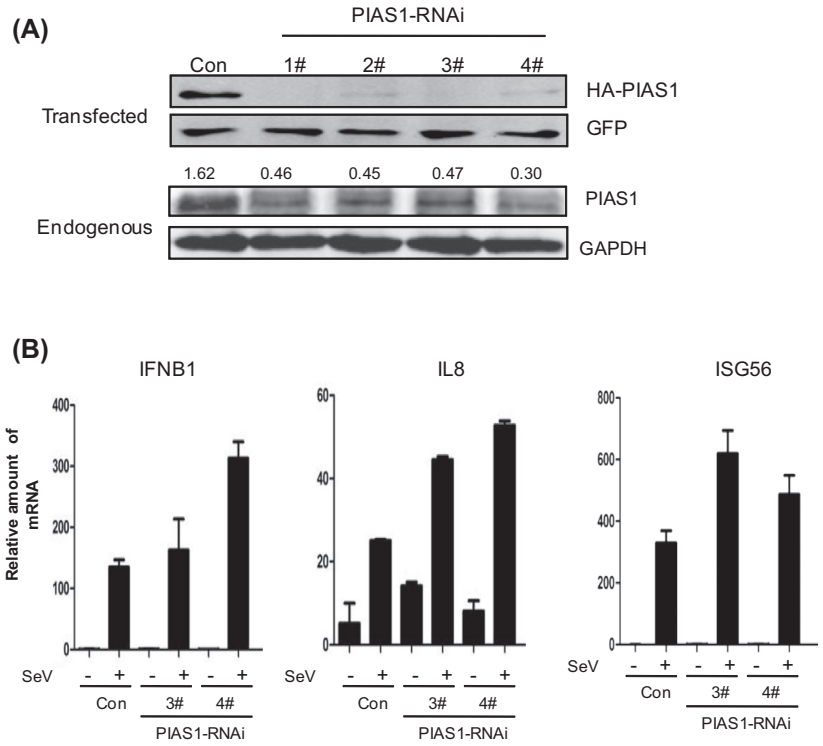

Fig. 2. Effects of PIAS1 knockdown on virus-induced signaling. (A) Effects of PIAS1 shRNA plasmids on the expression of overexpressed or endogenous PIAS1. HEK293 cells (1.0×10^5) were seeded on 24-well plates and were transfected with 1 μg HA-tagged PIAS1, 1 μg pEGFP with control or PIAS1-shRNA plasmids (2 μg each) as indicated. Twenty-four hours after transfection, cell lysates were analyzed by Western blot with anti-HA or anti-GFP (upper panel). Total HEK293 cell lysates were blotted with anti-PIAS1 antibody. (B) Knockdown of PIAS1 expression increased SeV-induced expression of endogenous *IFNβ*, *ISG56* and *IL8*. HEK293 cells (1.0×10^5) were seeded on 24-well plates and were transfected with a control or the indicated PIAS1 shRNA plasmids (2 μg each). Twenty-four hours after transfection, cells were infected with SeV or left uninfected for twelve hours before Real-time PCR analysis of the expression of the indicated mRNAs.

3.3. PIAS1 regulates virus-triggered signaling at the IRF3 level

Recognition of viral RNA by RIG-I leads to the activation of downstream signaling molecules such as VISA, TBK1, IKKε, IRF3 (Zhao et al., 2007). We next sought to determine a step within the signaling cascade that PIAS1 targets. As shown in Fig. 3, ISRE and IFN-β promoter activity were activated by transfection of an active form of RIG-I (RIG-IN), VISA, TBK1, IKKε, and an activated form of IRF3 (IRF3/5D) respectively. In all cases, co-expression of PIAS1 reduced ISRE and IFN-β promoter activity in a dose-dependent manner (Fig. 3A & B). These results indicated that PIAS1 targeted at IRF3 or a downstream step from IRF3 phosphorylation. Additionally, we cotransfected PIAS1 with TRIF and found that PIAS1 inhibited TRIF-induced activation of ISRE and IFN-β promoter (Supplementary Fig. S3). This suggested that PIAS1 not only negatively regulated RLRs signaling but also TLRs signaling. Once activated, IRF3 is phosphorylated at Ser-396 and Ser-386 by kinase TBK1 or IKKε. Phosphorylated IRF3 forms homodimers and translocates to the nucleus (Lin et al., 1998). To test whether PIAS1 affects these critical events of IRF3 activation, HEK293T cells were transfected with Flag-tagged VISA, and Flag-tagged IRF3 along with HA-tagged PIAS1 and phosphorylated IRF3 was detected by anti-phospho-IRF3 antibody. Co-expression of VISA increased the phosphorylation of IRF3 at Ser-396 and this was not affected by PIAS1 (Fig. 3C). We also detected whether PIAS1 affects virus-induced nuclear translocation of IRF3. Cells transfected with HA-tagged PIAS1 or empty vector were infected with Sendai virus and stained with anti-IRF3 antibody at six hours post infection. After infection, IRF3 translocated to the nucleus irrespective of PIAS1 transfection (Fig. 3D). These data indicate that PIAS1 inhibits type I IFN promoter activation without interfering with the phosphorylation and nuclear translocation of IRF3.

3.4. LXXLL motif in the SAP domain is not involved in the inhibition of the type I IFN transcription

PIAS family members have been reported to inhibit IFN-mediated activation of IFN stimulated genes (ISGs) by preventing STAT-dependent activation of ISRE promoter activity (Shuai and Liu, 2005). It was reported that the conserved LXXLL motif in the N-terminal SAP domain of PIASy critically contributes to the inhibition of STAT1-dependent transcription (Liu et al., 2001). To exclude the possibility that the inhibitory effect on type I IFN promoter was due to PIAS1 inhibition of STAT1 activity, we generated a mutant PIAS1 in which all three leucine residues in the LXXLL motif were substituted to alanine (Fig. 4A). We also tested the effects of WT and PIAS1/mSAP on IFN-β-stimulated ISRE promoter activity and found that PIAS1/mSAP abolished the ability to inhibit ISRE promoter activity (Fig. 4B). This indicated that the LXXLL motif was very important for the inhibitory function of PIAS1 on STAT1-dependent signaling. In contrast, activation of ISRE and IFN-β promoter activities induced by Sendai virus was similarly inhibited by PIAS1/mSAP and WT PIAS1 (Fig. 4C). Consistently, the transcription of *IFNB1* was at the similar level in PIAS1/mSAP and WT PIAS1 transfected cells (Fig. 4D). These suggest that the LXXLL motif of PIAS1 does not contribute to virus-induced type I IFN signaling and PIAS1 has a direct effect on this process.

3.5. The inhibitory activity of PIAS1 on virus triggered signaling is independent of its SUMO E3 ligase activity

PIAS family members are well-documented to act as SUMO E3 ligases for multiple substrates. To study whether SUMO E3 ligase activity of PIAS1 is required for the inhibition of type I IFN

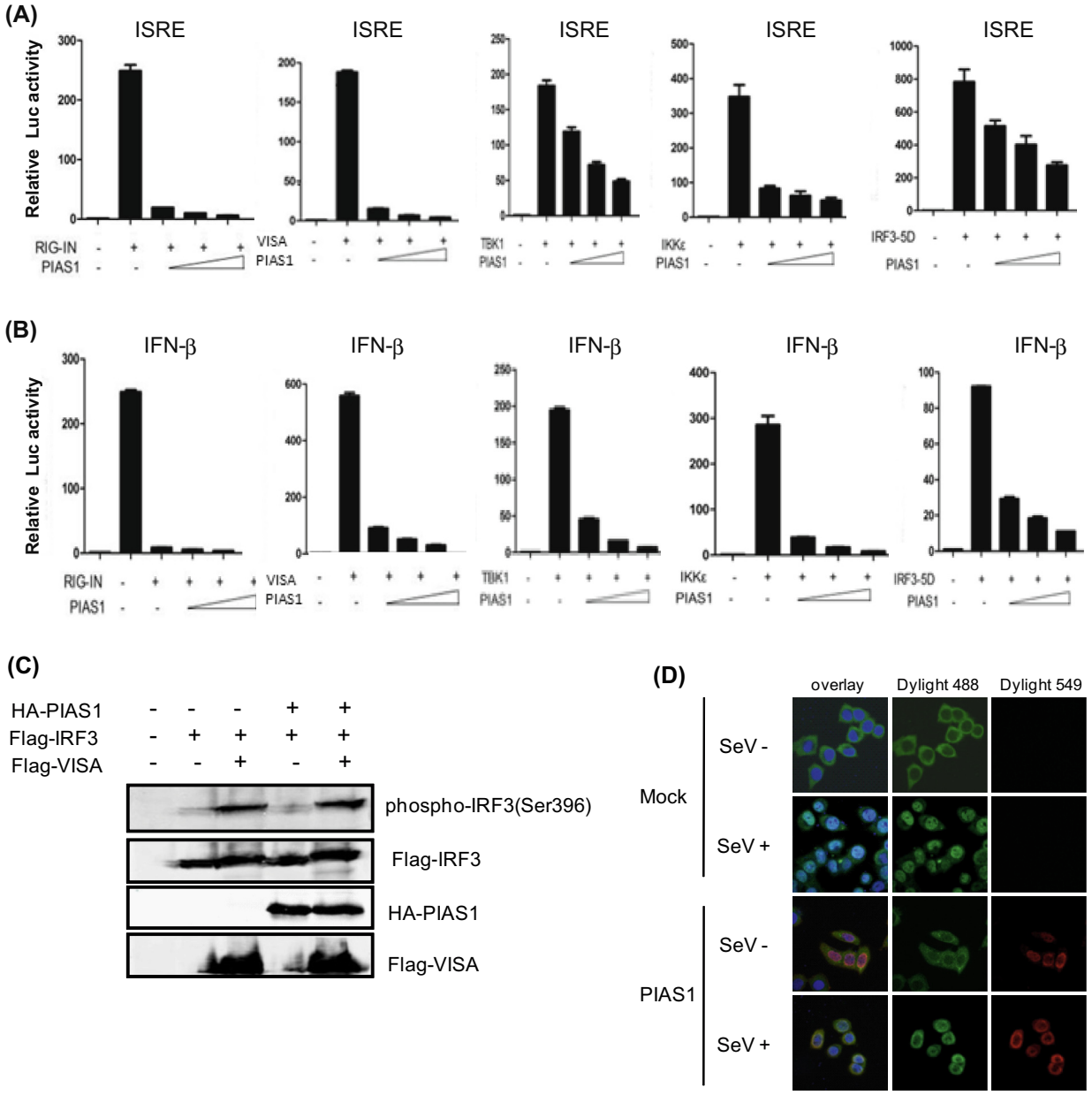

Fig. 3. PIAS1 targets at or downstream of IRF3. (A) Effects of PIAS1 on ISRE activation by various signaling components in a dose-dependent manner. (B) Effects of PIAS1 on IFN-β activation by various signaling components in a dose-dependent manner. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with indicated signaling molecules, IFN-β or ISRE luciferase reporter and pRL-SV40 plasmid, and increasing doses of PIAS1 for twenty-four hours. The luciferase activities were quantified by normalizing with *Renilla* luciferase activities. (C) Effects of PIAS1 on IRF3 phosphorylation. HEK293T cells (2.0×10^5) were seeded on 12-well plates and were transfected the next day with mock control or PIAS1(0.5 μg) combined with IRF3 (0.5 μg) or/and MAVS expressing vector (0.5 μg). Cell lysates were blotted with anti-IRF3 pSer 396 antibody (1:500). (D) HEK293 cells (9×10^4) were seeded on 6-well plates and the next day cells were transfected with mock control or PIAS1. After twenty-four hours cells were infected with SeV or left untreated for six hours. Then fixed cells were stained with anti-IRF3 antibody and anti-PIAS1 antibody, then cells were viewed on the confocal microscope.

transcription, we constructed a mutant PIAS1 in which the third cysteine residue of the RING-like domain was substituted by serine (PIAS1/C350S) (Fig. 5A). Compared to WT PIAS1, PIAS1/C350S mutant lost the SUMO E3 activity (Fig. 5B). Furthermore, using p53 as the specific substrate, PIAS1/C350S also displayed abolished ability to SUMOylate p53 (data not shown). Reporter assays showed that SeV-induced activation of ISRE and IFN-β promoter activities were inhibited by PIAS1/C350S to a similar degree as WT PIAS1 (Fig. 5C). The transcription of *IFNB1* induced by SeV infection was inhibited by PIAS1/C350S as well (Fig. 5D). These data indicate that PIAS1 inhibits virus-induced type I IFN induction independent of its SUMO E3 ligase activity.

3.6. PIAS1 interacts with IRF3 and inhibits the DNA binding activity of IRF3

Once activated, IRF3 translocates to the nucleus and binds to IRF3-responsive elements containing sequences such as in IFN-β promoter regions to activate gene transcription (Wang et al., 2009b). Previous studies reported that PIAS1 can block the DNA binding activity of transcription factor STAT1 and p65 (Liu et al., 1998; Liu et al., 2005). As mentioned above, PIAS1 may act at IRF3 level, so we first tested that if PIAS1 can interact with IRF3, as shown in Fig. 6A, PIAS1 can interact with IRF3 (Fig. 6A, left panel). Intriguingly, this interaction was enhanced by Sendai virus

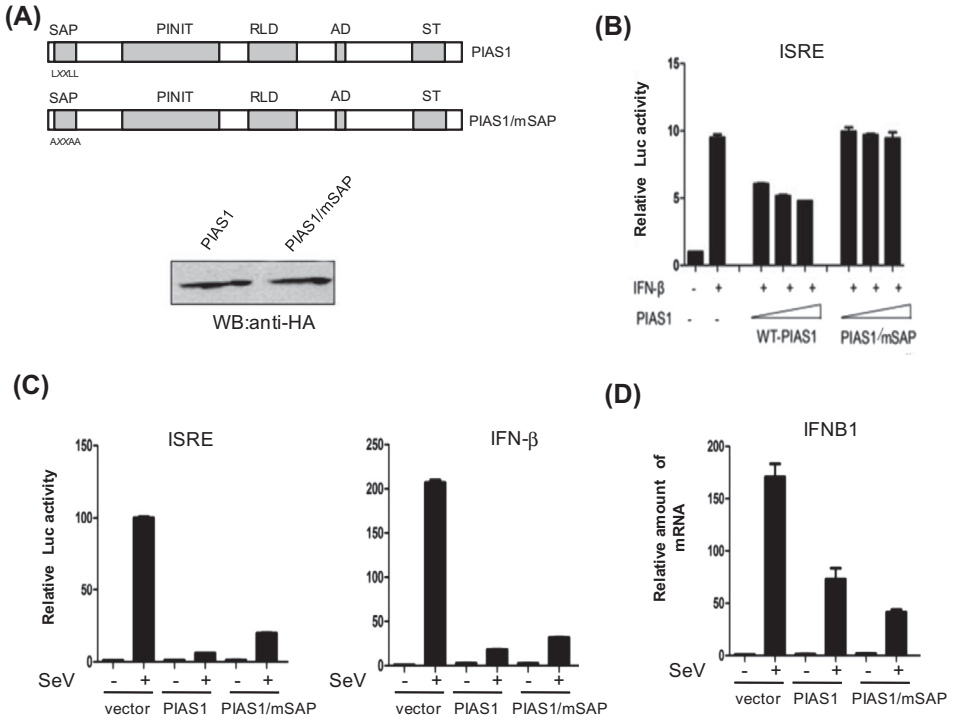

Fig. 4. *LXXLL* motif of PIAS1 is not involved in the regulation of SeV-induced signaling. (A) Schematic diagram of the construction of PIAS1/mSAP mutant (upper panel). Its expression was detected by western blot and shown in the lower panel. (B) PIAS1/mSAP abolished the ability to inhibit IFN-β induced ISRE activation. HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with ISRE luciferase reporter, and increasing doses of PIAS1 for twenty-four hours, then left treated or untreated with IFN-β (1000 U/ml) for six hours. The luciferase activities were quantified by normalizing with *Renilla* luciferase activities. (C) PIAS1/mSAP inhibited SeV-induced ISRE and IFN-β promoter activation. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control, PIAS1 or PIAS1/mSAP expressing vector (1 μg each), together with ISRE or IFN-β reporter vector. Twenty-four hours later, cells were infected with SeV or left uninfected for twenty hours before luciferase assays were performed. (D) PIAS1/mSAP inhibited SeV-induced transcription of *IFNB1*. HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control, PIAS1 or PIAS1/mSAP expressing vector (1 μg each). Twenty-four hours later, cells were left uninfected or infected with SeV for nine hours. Real-time PCR analysis of the *IFNB1* mRNA expression was shown.

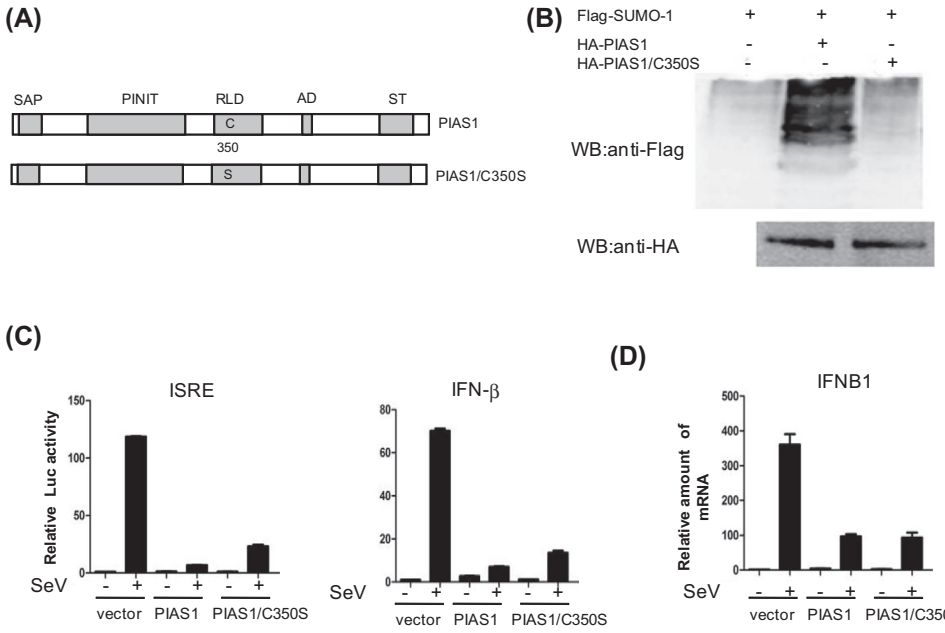

Fig. 5. PIAS1 inhibited virus triggered signaling independent of its SUMO E3 ligase activity. (A) Schematic diagram of the construction of PIAS1/C350S mutant. (B) In vivo SUMOylation assay. HEK293T cells were transiently transfected with Flag-tagged SUMO-1 (0.5 μg), together with HA-tagged PIAS1 or PIAS1/C350S (0.5 μg each). Whole cell lysates were analyzed by western blot with anti-Flag or anti-HA. (C) PIAS1/C350S inhibited SeV-induced ISRE and IFN-β promoter activation. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control, PIAS1 or PIAS1/C350S expressing vector (1 μg each), together with ISRE or IFN-β reporter vector. Twenty-four hours later, cells were infected with SeV or left uninfected for twenty hours before luciferase assays were performed. (D) PIAS1/C350S inhibited SeV-induced transcription of *IFNB1*. HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control, PIAS1 or PIAS1/C350S expressing vector (1 μg each). Twenty-four hours later, cells were left uninfected or infected with SeV for nine hours. Real-time PCR analysis of the *IFNB1* mRNA expression was shown.

infection (Fig. 6A, right panel). Then we tested if PIAS1 also inhibited the DNA binding activity of IRF3. By ChIP assay, in vector-transfected cells, Sendai virus infection increased the amounts of IRF3 associated with the IFN-β promoter region at eight hours post-infection, whereas the binding was attenuated in the presence of PIAS1 (Fig. 6B). The DNA binding activity of a positive control, RNA polymerase II was not affected by PIAS1 (Fig. 6B). This suggests that PIAS1 can directly inhibit the DNA binding activity of IRF3. IRF7 represents another important transcription factor, which plays important role in virus-induced positive feedback signaling. We also tested the interaction between PIAS1 and IRF7 and found that PIAS1 can interact with IRF7 and inhibited IRF7-6D induced ISRE and IFN-β activation (Supplementary Fig. S4A & B).

3.7. C-terminal of PIAS1 is critical for its suppressive function on virus-induced type I IFN signaling

Next, we tried to investigate which domain of PIAS1 is required for the interaction with IRF3 and confers PIAS1 the inhibitory functions on virus-induced type I IFN signaling. We constructed a series of PIAS1 mutants including C-terminal truncated mutants of PIAS1 and a mutant devoid of the SIM motif (PIAS1/ΔSIM) (Fig. 7A & B). Recently, a short splicing of PIAS1 was reported, which encodes amino acids 1–311 (Wang et al., 2009c). We also constructed the expressing vector for it and designated it as PIAS1-S in this manuscript (Fig. 7A & B). First we found that PIAS1/C350A still interacted with IRF3 (data not shown). This is consistent with its inhibitory function. Moreover, as shown in Fig. 7C, PIAS1/1-520 still retained the ability to interact with IRF3 and has the similar inhibitory function on VISA-induced ISRE activation to WT PIAS1 (Fig. 7C & D). When the deletion was extended further, PIAS1/1-460 lost the ability to interact with IRF3 (Fig. 7C). Reporter assays showed the suppressive effects on VISA-induced activation of ISRE and IFN-β promoter activities were attenuated by PIAS1/1-460 compared to WT PIAS1 (Fig. 7D). From amino acid 460–520, there contains a cluster of acidic amino acids (AD). However, PIAS1 with SIM deletion still suppressed VISA-induced ISRE activation (Fig. 7D) and interacted with IRF3 (data not shown). These data together suggest that the C-terminal region of PIAS1 around AD is responsible for its suppressive function on virus-induced type I IFN signaling.

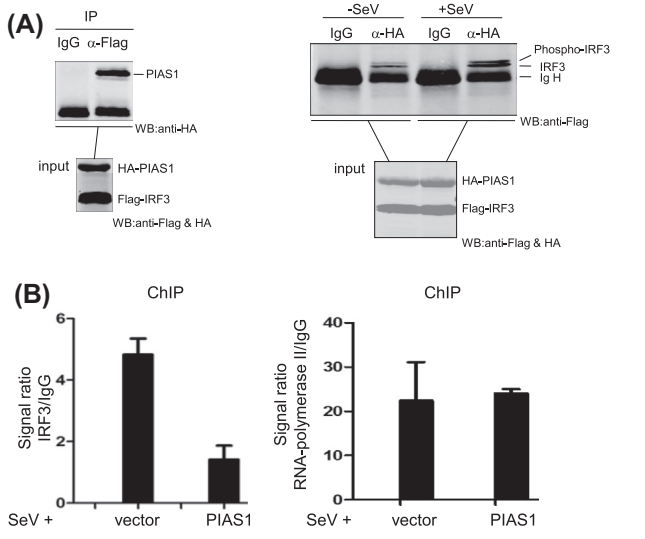

Fig. 6. PIAS1 interacted with IRF3 and blocked the DNA binding activity of IRF3. (A) PIAS1 interacted with IRF3. HEK293T cells were transfected with Flag-tagged IRF3 (10 μg) and HA-tagged PIAS1 (10 μg). Coimmunoprecipitation was performed with mouse IgG or anti-Flag antibody and the membrane was blotted with anti-HA antibody (left panel). Then the coimmunoprecipitation were also performed after with or without Sendai virus infection (right panel). Coimmunoprecipitation was performed with mouse IgG or anti-HA antibody and the membrane was blotted with anti-Flag antibody (B) ChIP analysis of IRF3 binding to the IFN-β promoter. HEK293T cells were transfected with mock or PIAS1 expressing vector (10 μg each), then left infected with SeV for six hours. Then ChIP assay was performed as described in materials and methods. Real-time PCR analysis of the relative binding of IRF3 to the IFN-β promoter.

4. Discussion

PIAS family members are important brakes in restraining inflammation. Different PIAS members display specific or redundant roles in this process. They may also cooperate in the regulation. In the present study, we investigated the contribution of PIAS1 to the regulation of type I IFN transcription. Overexpression of PIAS1 inhibited virus-triggered type I IFN expression. Knockdown of PIAS1 displayed enhanced production of interferon. Like PIASy, endogenous PIAS1 is uniformly expressed in the nucleus (Alm-Kristiansen et al., 2011). Reporter assays showed that PIAS1 may act at the downstream step from IRF3 phosphorylation. As expected, PIAS1 did not affect the phosphorylation and nuclear translocation of IRF3. These data suggest that PIAS1 may function exclusively in the nucleus. Our further studies demonstrate that PIAS1 can directly block the DNA binding activity of IRF3.

Previous research focused on the regulatory roles of PIAS1 in interferon mediated activation of IFN stimulated genes (ISGs) by preventing STAT1-dependent activation of ISRE promoter activity (Liu et al., 1998). To exclude the indirect effects of PIAS1 on inhibition of STAT1 activity, we constructed PIAS1 with a mutation at the *LXXLL* motif. It was reported that the conserved *LXXLL* motif in the N-terminal SAP domain of PIASy critically contributed to the inhibition of STAT1-dependent transcription (Liu et al., 2001). Here we demonstrated that this motif was also very important for the inhibitory function of PIAS1 on STAT1-dependent signaling. We found that PIAS1/mSAP mutant lost the ability to inhibit IFN-stimulated ISG transcription. However, it still retained the ability to inhibit type I IFN transcription. This suggests that the SAP domain independent inhibition of ISRE activation may occur at the early stage of virus infection. PIAS1 is not only an important regulator in the late IFN stimulated amplifying stage, but also a crucial participant to control the virus induced early signaling stage.

PIAS family members possess SUMO-E3-ligase activity. To test whether SUMO-E3-ligase activity of PIAS1 is involved in the negative regulation, we constructed PIAS1/C350S mutant which lost the SUMO E3 ligase activity. Using this mutant, we observed that it could inhibit virus induced type I IFN signaling to the similar extent as WT PIAS1. This implies that PIAS1 negatively regulates virus triggered signaling in a SUMO-E3 ligase independent manner.

Previous studies showed that PIAS1 functions by blocking the DNA-binding activity of STAT1 and p65 on gene promoters (Liu et al., 1998, 2005). We also tested whether PIAS1 affected the DNA binding activity of IRF3. By coimmunoprecipitation and ChIP assay, we found that PIAS1 can interact with IRF3 and overexpression of PIAS1 inhibited the DNA binding activity of IRF3 induced by Sendai virus infection. Interestingly, we also found that PIAS1 interact with IRF7 and inhibited IRF7-6D induced ISRE and IFN-β activation. In our early studies and others, we observed PIASy can act at both IRF3 and IRF7 level (Kubota et al., 2011; Zhang et al., 2004). It raised the possibility that they coordinate at the regulation of virus-induced signaling.

Domain mapping the critical regions of PIAS1 responsible for the interaction with IRF3 and the inhibitory functions of PIAS1 characterized the C-terminal region around a cluster of acidic amino acids are essential. Until now, the functional significance of the acidic cluster was not fully understood. PIASy was also reported that deletion of the acidic cluster resulted in the loss of PIASy

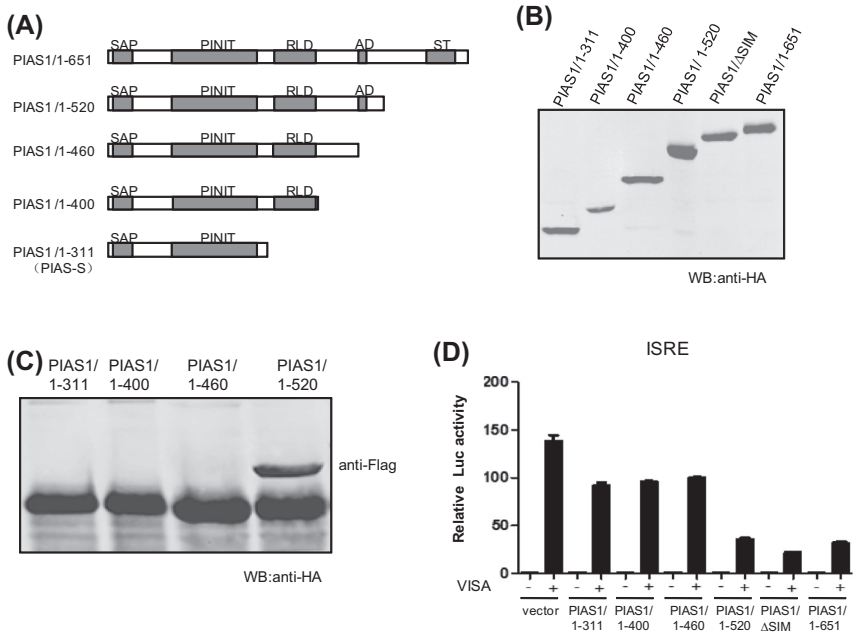

Fig. 7. C-terminal of PIAS1 is critical for inhibiting virus-induced type I IFN signaling. (A) Schematic structure of the full-length PIAS1 protein and the C-terminal truncated PIAS1 protein. Conserved SAP domain, PINIT domain, RING-like domain (RLD), acidic domain (AD), and serine/threonine-rich domain (S/T) are shown on top. (B) The expression of constructed C-termianl truncated PIAS1 and PIAS1/ΔSIM. (C) Coimmunoprecipitation of C-terminal truncated PIAS1 and IRF3.Coimmunoprecipitation was performed with mouse IgG or anti-Flag antibody and the membrane was blotted with anti-HA antibody. (D) PIAS1/1-460 abolished the ability to inhibit SeV-induced ISRE and IFN-β promoter activation. Human HEK293T cells (1.2×10^5) were seeded on 24-well plates and were transfected the next day with mock control, PIAS1 or PIAS1 mutants expressing vector (1 μg each), together with VISA and ISRE reporter vector. Twenty-four hours later, luciferase assays were performed.

function, leading to the loss of inhibtion of IFN transcription (Kubota et al., 2011).

PIAS1 and PIASy have specific and redundant roles in the induction of NF-κB and STAT1 target genes and that the cooperative action of PIAS1 and PIASy controls the specificity and magnitude of NF-κB and STAT1 mediated gene activation (Tahk et al., 2007). Previously we reported that PIASy negatively regulates TLR-3 dependent virus signaling (Zhang et al., 2004). This was also confirmed by another group (Kubota et al., 2011). When knockdown of endogenous PIAS1 expression resulted in enhanced IFN-β production. But this effect is very limited. We presume this is due to the redundant roles of other PIAS family members such as PIASy.

Upon stimulation by proinflammatory stimuli, such as TNF-α or LPS, PIAS1 becomes rapidly phosphorylated on Ser90, an event that triggers the recruitment of PIAS1 to the promoters of NF-κB and STAT1 target genes, resulting in the inhibition of the promoter-binding activity of NF-κB and STAT1. Inhibiton of κB kinase α (IKKα) has been identified as the kinase responsible for PIAS1 Ser90 phosphorylation that is induced by proinflammatory gene induction (Liu et al., 2007). To test that if Ser90 is also very critical for the inhibitory role of PIAS1 in virus induced signaling. We constructed PIAS1/S90D and PIAS1/S90A mutants. In reporter assays, we found that compared with WT PIAS1, both mutants displayed similar inhibitory functions (Supplementary Fig. S5). This implies that Ser90 phosphorylation of PIAS1 is dispensable for the inhibitory function of PIAS1 on virus induced type I IFN signaling.

IRF3 is the key target for negative regulation during virus infection. Some negative regulators target IRF3 for post-transcriptional modifications. Ubiquitination is the major modification for IRF3, which mainly leads to the proteasome-mediated degradation of IRF3. For example, Pin1 and RBCK1 promote the ubiquitination of IRF3 and decrease IRF3 protein abundance (Baccala et al., 2007; Saitoh et al., 2006). In addition, mouse IRF3 is reported to be SUMOylated at K152, and this SUMOylation negatively regulates IRF3 transcriptional activity (Kubota et al., 2008). A recent study reports that K87 is the conserved site for both SUMOylation and ubiquitination. A deSUMOylating enzyme Sentrin/SUMO-specific protease (SENP)2 directly deSUMOylates IRF3 and conditions it for ubiquitination and degradation (Ran et al., 2011). MafB, a member of Maf transcription factors, which does not promote the post-modifications of IRF3, antagonizes antiviral responses by blocking recruitment of coactivator CBP to IRF3 (Kim and Seed, 2010). The study in PIASy showed that although PIASy inhibited virus induced signaling independent of SUMO E3 ligase activity, this inhibitory role still was dependent on a mechanism involving the conjugation of SUMO (Kubota et al., 2011). Therefore, at present, we cannot exclude the possibility that PIAS1 may also function dependent on SUMOylation of itself or other related molecules. In the present study, by ChIP assay, we found that PIAS1 can markedly influence the DNA binding activity of IRF3. To our knowledge, this is the first study that reports the DNA binding activity of IRF3 is targeted for negative regulation. Taken together, these suggest that IRF3, the key transcription factor in innate immunity is modulated at multiple levels to ensure proper extent of responses occur.

In conclusion, we show here PIAS1 is an important regulator of virus triggered type I IFN production. It represses by inhibiting the DNA binding activity of transcription factor IRF3. Further investigation is needed to delineate the delicate mechanisms responsible for the inhibitory roles of PIAS1 in virus induced type I IFN transcription.

Acknowledgments

We thank Dr. Laura Crisponi for myc-tagged PIAS1, Dr. Takashi Fujita for RIG-I expressing vector, Dr. Tom Maniatis for TBK1 and IKKε vector, Dr. Zhijian Chen for MAVS(VISA) vector. We thank Dr. Ping-Zhang Wang and Da-Long Ma for PIAS1-S clone. We also thank Drs. Hong-Bing Shu, Hong Tang, Dan-Ying Chen for other signaling molecule vectors and reagents.

This study received supports from the National Basic Research Program of China (2010CB945300, 2011CB946103).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.09.001.

References

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Alm-Kristiansen, A.H., Lorenzo, P.I., Molvaersmyr, A.K., Matre, V., Ledsaak, M., Saether, T., Gabrielsen, O.S., 2011. PIAS1 interacts with FLASH and enhances its co-activation of c-Myb. Mol. Cancer 10, 21.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., Randall, R.E., 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc. Natl. Acad. Sci. USA 101, 17264–17269.

Baccala, R., Hoebe, K., Kono, D.H., Beutler, B., Theofilopoulos, A.N., 2007. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. Nat. Med. 13, 543–551.

Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., Shuai, K., 1997. Specific inhibition of Stat3 signal transduction by PIAS3. Science 278, 1803–1805.

Jackson, P.K., 2001. A new RING for SUMO: wrestling transcriptional responses into nuclear bodies with PIAS family E3 SUMO ligases. Genes Dev. 15, 3053–3058.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., Yamaguchi, O., Otsu, K., Tsujimura, T., Koh, C.S., Reis e Sousa, C., Matsuura, Y., Fujita, T., Akira, S., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101–105.

Kawai, T., Akira, S., 2006. Innate immune recognition of viral infection. Nat. Immunol. 7, 131–137.

Kim, H., Seed, B., 2010. The transcription factor MafB antagonizes antiviral responses by blocking recruitment of coactivators to the transcription factor IRF3. Nat. Immunol. 11, 743–750.

Komuro, A., Bamming, D., Horvath, C.M., 2008. Negative regulation of cytoplasmic RNA-mediated antiviral signaling. Cytokine 43, 350–358.

Kubota, T., Matsuoka, M., Chang, T.H., Tailor, P., Sasaki, T., Tashiro, M., Kato, A., Ozato, K., 2008. Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. J. Biol. Chem. 283, 25660–25670.

Kubota, T., Matsuoka, M., Xu, S., Otsuki, N., Takeda, M., Kato, A., Ozato, K., 2011. PIASy inhibits virus-induced and interferon-stimulated transcription through distinct mechanisms. J. Biol. Chem. 286, 8165–8175.

Liang, M., Melchior, F., Feng, X.H., Lin, X., 2004. Regulation of Smad4 sumoylation and transforming growth factor-beta signaling by protein inhibitor of activated STAT1. J. Biol. Chem. 279, 22857–22865.

Lin, R., Heylbroeck, C., Pitha, P.M., Hiscott, J., 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol. Cell. Biol. 18, 2986–2996.

Liu, B., Shuai, K., 2008. Targeting the PIAS1 SUMO ligase pathway to control inflammation. Trends Pharmacol. Sci. 29, 505–509.

Liu, B., Liao, J., Rao, X., Kushner, S.A., Chung, C.D., Chang, D.D., Shuai, K., 1998. Inhibition of Stat1-mediated gene activation by PIAS1. Proc. Natl. Acad. Sci. USA 95, 10626–10631.

Liu, B., Gross, M., ten Hoeve, J., Shuai, K., 2001. A transcriptional corepressor of Stat1 with an essential LXXLL signature motif. Proc. Natl. Acad. Sci. USA 98, 3203–3207.

Liu, B., Mink, S., Wong, K.A., Stein, N., Getman, C., Dempsey, P.W., Wu, H., Shuai, K., 2004. PIAS1 selectively inhibits interferon-inducible genes and is important in innate immunity. Nat. Immunol. 5, 891–898.

Liu, B., Yang, R., Wong, K.A., Getman, C., Stein, N., Teitell, M.A., Cheng, G., Wu, H., Shuai, K., 2005. Negative regulation of NF-kappaB signaling by PIAS1. Mol. Cell. Biol. 25, 1113–1123.

Liu, B., Yang, Y., Chernishof, V., Loo, R.R., Jang, H., Tahk, S., Yang, R., Mink, S., Shultz, D., Bellone, C.J., Loo, J.A., Shuai, K., 2007. Proinflammatory stimuli induce IKKalpha-mediated phosphorylation of PIAS1 to restrict inflammation and immunity. Cell 129, 903–914.

Nishida, T., Yasuda, H., 2002. PIAS1 and PIASxalpha function as SUMO-E3 ligases toward androgen receptor and repress androgen receptor-dependent transcription. J. Biol. Chem. 277, 41311–41317.

O'Neill, L.A., 2008. When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. Immunity 29, 12–20.

Ran, Y., Liu, T.T., Zhou, Q., Li, S., Mao, A.P., Li, Y., Liu, L.J., Cheng, J.K., Shu, H.B., 2011. SENP2 negatively regulates cellular antiviral response by deSUMOylating IRF3 and conditioning it for ubiquitination and degradation. J. Mol. Cell Biol. 3, 283–292.

Roth, W., Sustmann, C., Kieslinger, M., Gilmozzi, A., Irmer, D., Kremmer, E., Turck, C., Grosschedl, R., 2004. PIASy-deficient mice display modest defects in IFN and Wnt signaling. J. Immunol. 173, 6189–6199.

Saitoh, T., Tun-Kyi, A., Ryo, A., Yamamoto, M., Finn, G., Fujita, T., Akira, S., Yamamoto, N., Lu, K.P., Yamaoka, S., 2006. Negative regulation of interferon-regulatory factor 3-dependent innate antiviral response by the prolyl isomerase Pin1. Nat. Immunol. 7, 598–605.

Schmidt, D., Muller, S., 2002. Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. Proc. Natl. Acad. Sci. USA 99, 2872–2877.

Schmidt, D., Muller, S., 2003. PIAS/SUMO: new partners in transcriptional regulation. Cell. Mol. Life Sci. 60, 2561–2574.

Shuai, K., 1999. The STAT family of proteins in cytokine signaling. Prog. Biophys. Mol. Biol. 71, 405–422.

Shuai, K., 2000. Modulation of STAT signaling by STAT-interacting proteins. Oncogene 19, 2638–2644.

Shuai, K., Liu, B., 2003. Regulation of JAK-STAT signalling in the immune system. Nat. Rev. Immunol. 3, 900–911.

Shuai, K., Liu, B., 2005. Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat. Rev. Immunol. 5, 593–605.

Tahk, S., Liu, B., Chernishof, V., Wong, K.A., Wu, H., Shuai, K., 2007. Control of specificity and magnitude of NF-kappa B and STAT1-mediated gene activation through PIASy and PIAS1 cooperation. Proc. Natl. Acad. Sci. USA 104, 11643–11648.

Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14.

Wang, J., Hu, Y., Deng, W.W., Sun, B., 2009a. Negative regulation of Toll-like receptor signaling pathway. Microbes Infect. 11, 321–327.

Wang, J.T., Doong, S.L., Teng, S.C., Lee, C.P., Tsai, C.H., Chen, M.R., 2009b. Epstein-Barr virus BGLF4 kinase suppresses the interferon regulatory factor 3 signaling pathway. J. Virol. 83, 1856–1869.

Wang, P., Yu, P., Gao, P., Shi, T., Ma, D., 2009c. Discovery of novel human transcript variants by analysis of intronic single-block EST with polyadenylation site. BMC Genomics 10, 518.

Wong, K.A., Kim, R., Christofk, H., Gao, J., Lawson, G., Wu, H., 2004. Protein inhibitor of activated STAT Y (PIASy) and a splice variant lacking exon 6 enhance sumoylation but are not essential for embryogenesis and adult life. Mol. Cell. Biol. 24, 5577–5586.

Xu, L.G., Wang, Y.Y., Han, K.J., Li, L.Y., Zhai, Z., Shu, H.B., 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol. Cell 19, 727–740.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S., 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640–643.

Yoneyama, M., Fujita, T., 2009. RNA recognition and signal transduction by RIG-I-like receptors. Immunol. Rev. 227, 54–65.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat. Immunol. 5, 730–737.

Zhang, J., Xu, L.G., Han, K.J., Wei, X., Shu, H.B., 2004. PIASy represses TRIF-induced ISRE and NF-kappaB activation but not apoptosis. FEBS Lett. 570, 97–101.

Zhao, T., Yang, L., Sun, Q., Arguello, M., Ballard, D.W., Hiscott, J., Lin, R., 2007. The NEMO adaptor bridges the nuclear factor-kappaB and interferon regulatory factor signaling pathways. Nat. Immunol. 8, 592–600.