PIASy represses TRIF-induced ISRE and NF-κB activation but not apoptosis

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Abstract The TIR domain-containing adapter protein TRIP is critically involved in TLR3-induced IFN-β production through activation of NF-κB and ISRE. In addition, TRIF also induces apoptosis when overexpressed in 293 cells. In this report, we demonstrate that PIASy, a member of the PIAS SUMO-ligase family, interacts with TRIP, IRF-3 and IRF-7. In reporter gene assays, PIASy dramatically inhibits TRIF-induced NF-κB, ISRE and IFN-β activation but not TRIF-induced apoptosis. Furthermore, PIASy also inhibits IRF-3, IRF-7 and Sendai virus-induced ISRE activation. Our results suggest that PIASy is an inhibitor of TRIF-induced ISRE and NF-κB activation but not apoptosis.

Keywords: PIASy; TRIP; ISRE; IFN-β; NF-κB

1. Introduction

The Toll/interleukin-1 receptor (TIR) family members are evolutionary conserved proteins that are critically involved in innate immunity [1–3]. This family of receptors contains a conserved cytoplasmic TIR domain that is required for their signaling leading to activation of transcription factors NF-κB, ATF-2/c-Jun and IRFs, and subsequent induction of various chemokines and cytokines that are involved in host defense against the pathogens [1–3].

The TIR family receptors signal through conserved pathways. Upon ligand stimulation, TIR family receptors recruit TIR domain-containing adapter proteins to the receptor signaling complexes. So far, there are four TIR domain-containing adapter proteins, including MyD88, TIRAP/Mal, TRIF/TICAM and TIRP/TRAM, which have been cloned [4–10]. These proteins have distinct and overlapping roles in TIR signaling.

Among the TIR domain-containing adapters, TRIF is unique in that it can activate both NF-κB and ISRE. A dominant negative mutant of TRIF inhibits both TLR3-mediated NF-κB and ISRE activation [8,9]. Recently, TRIF-deficient mice were produced by gene knock-out and chemical mutagenesis approaches [11,12]. These studies indicated that TRIF-deficient mice were defective in both TLR3- and TLR4-mediated expression of IFN-β. It has been revealed that TRIF activates NF-κB through a TRAF6-dependent pathway and activates ISRE through IRF-3 and IRF-7 [13–16]. Two serine/threonine protein kinases, IKKε and TBK1, have been shown to be involved in phosphorylation and activation of IRF-3 and IRF-7 [14–16]. Recently, it has also been shown that TRIF can induce apoptosis through a RIP-FADD-caspase-8-dependent and mitochondria-independent pathway [16].

Protein inhibitors of activated STAT (PIAS) family members were originally identified as inhibitors of STAT proteins. In mammals, five members (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy) have been identified [17]. PIAS1 and PIAS3 have been shown to bind and inhibit STAT1 and STAT3 DNA-binding activities, respectively [17,18]. PIASxα and PIASxβ are probably derived from alternatively spliced mRNA products of the same gene. PIASxα interacts with the androgen receptor and regulates its activity [19,20]. PIASxβ interacts with the homeodomain protein Msx2 [21]. PIASy can inhibit STAT1-, Smad-, GATA-2, c-Myb or androgen receptor-mediated transcriptional responses without blocking their DNA-binding activities, [22–26] and repress LEF1 activity by sequestration into nuclear bodies [27]. In addition, the PIAS family members have been shown to act as SUMO-E3 ligases, which promote covalent addition of a small ubiquitin-related modifier to a target protein [19,20,27].

In this paper, we investigated the roles of one PIAS family member, PIASy, on TRIF signaling. Our findings suggest that PIASy negatively regulates TRIF-induced NF-κB and ISRE activation but not TRIF-induced apoptosis.

2. Materials and methods

2.1. Reagents

Human embryonic kidney 293 cells and Sendai viruses were purchased from ATCC (Manassas, VA). The monoclonal antibodies against the FLAG and HA epitopes were purchased from the Sigma company (St. Louis, MO).

2.2. Constructs

ISRE luciferase reporter construct, pFR-luciferase and pFA2-c-Jun vectors were obtained from Stratagene (La Jolla, CA). NF-κB luciferase reporter construct (Dr. Gary Johnson, University of Colorado Health Sciences Center) and mammalian expression plasmid for FLAG-TRAF6 (David Goeddel, Tualrik Inc.) were provided by the indicated investigators; IFN-β luciferase reporter construct, and mammalian expression plasmids for FLAG- or HA-tagged TRIF, IRF-3 and IRF-7 were previously described [5,16]. Mammalian expression plasmid for Flag-tagged PIASy was constructed by PCR.

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2.3. Cell transfection and reporter gene assays

293 cells (2×10^5) were seeded on 6-well (35-mm) dishes and were transfected the following day by the standard calcium phosphate precipitation [28]. Within the same experiment, each transfection was performed in triplicate, and where necessary, empty control plasmid was added to ensure that each transfection receives, the same amount of total DNA. To normalize for transfection efficiency, 0.3 μg of RSVβ-gal plasmid was added to each transfection. Luciferase reporter assays were performed using a Luciferase Assay Kit (BD PharMingen) and following the manufacturer's protocols, β-galactosidase activity was measured using the Galacto-Light Chemiluminescent Kit (TROPIX, Bedford, MA). Luciferase activities were normalized on the basis of β-galactosidase activities.

2.4. Co-immunoprecipitation and Western blot analysis

Transfected 293 cells from each 100-mm dish 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 and 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, 0.4 ml aliquots of lysates were incubated with 0.5 μg of the indicated monoclonal antibody or control mouse IgG and 20 μl of a 1:1 slurry of GammaBind G Plus Sepharose (Amersham Pharmacia, Piscataway, NJ) for at least 1 h. The sepharose beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS–PAGE and subsequent Western blot analyses were performed as described [5,16].

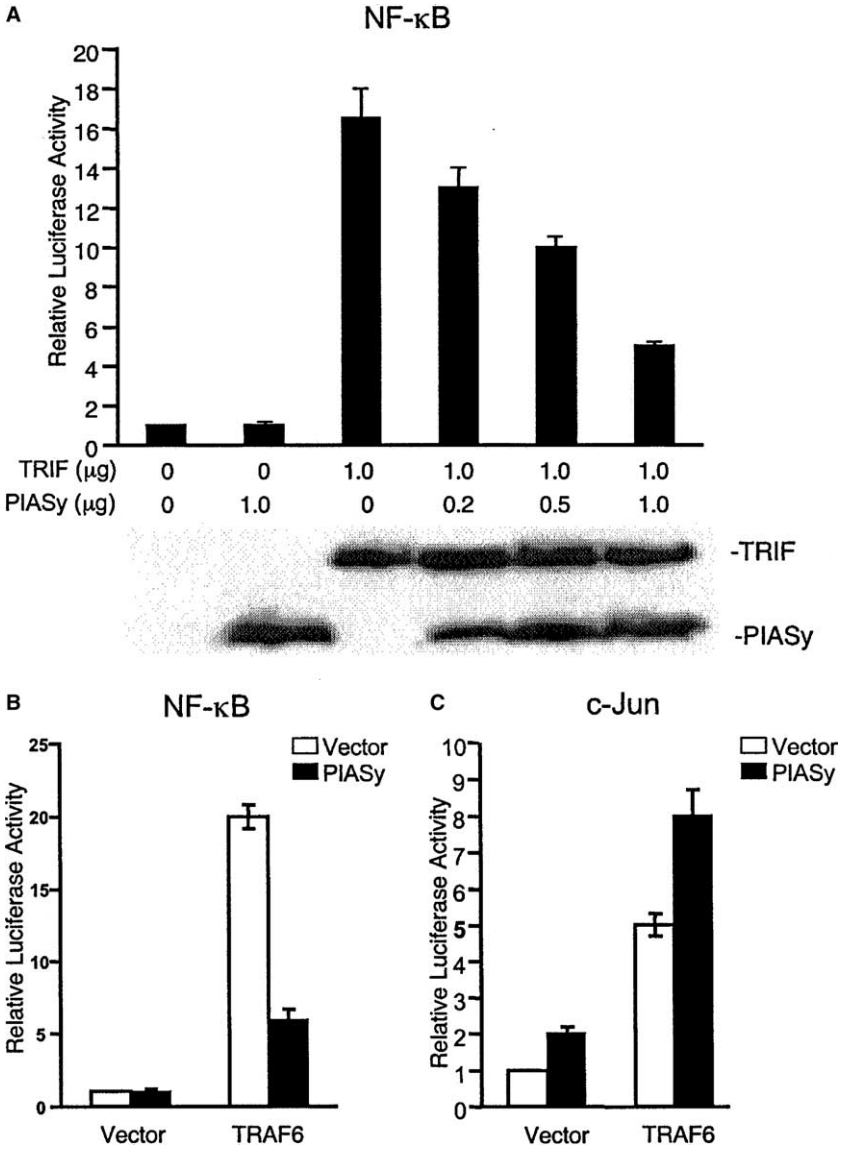

Fig. 1. PIASy inhibits TRIF- and TRAF6-induced NF-κB but not TRAF6-induced c-Jun activation. (A) PIASy inhibits TRIF-induced NF-κB activation in a dose-dependent manner. 293 cells ($\sim 2 \times 10^5$) were transfected with 0.3 μg of NF-κB-luciferase and 0.3 μg of RSV-β-gal plasmid and the indicated amounts of expression plasmids. Reporter gene assays were performed (upper panel). The expression of transfected proteins was detected by Western blot analysis (lower panel). (B) PIASy inhibits TRAF6-induced NF-κB activation. 293 cells ($\sim 2 \times 10^5$) were transfected with 0.3 μg of NF-κB-luciferase and 0.3 μg of RSV-β-gal plasmid and 1 μg of each of the indicated expression plasmids. (C) PIASy does not inhibit TRAF6-induced c-Jun activation. 293 cells were transfected with pFR-luciferase, pFA2-c-Jun, RSV-β-gal and the indicated plasmids. Luciferase reporter assays were performed 16 h after transfection. Data shown are averages and standard deviations of relative luciferase activities normalized based on β-gal activities. The experiments were repeated for at least three times with similar results.

2.5. Sendai virus infection

293 cells ($\sim 1 \times 10^5$) were seeded in 6-well dishes and transfected the next day with 0.5 μg ISRE reporter plasmid and 0.5 μg RSV-β-gal plasmid. Eighteen hours after transfection, cells were washed with medium lacking fetal calf serum (washing medium), then overlaid with washing medium containing Sendai virus at a multiplicity of infection of 10. After incubation at 37 °C for 60 min, non-adsorbed viruses were removed by repeated washing of the cells. Cells were then cultured in fetal calf serum-containing medium for 18 h before luciferase and β-galactosidase assays were performed.

2.6. Apoptosis assays

β-Galactosidase co-transfection assays for determination of cell death were performed. Briefly, 293 cells ($\sim 2 \times 10^5$) were seeded on 6-well (35 mm) dishes and were transfected the following day with 0.1 μg of pCMV-β-galactosidase plasmid and the indicated testing plasmids. Within the same experiment, each transfection was performed in triplicate, and where necessary, enough amount of empty control plasmid was added to keep each transfection receiving the same amount of total DNA. Approximately 24 h after transfection, the cells were stained with X-gal. The numbers of survived blue cells from five representative viewing fields were determined microscopically. Data shown are averages and standard deviations of one representative experiment in which each transfection has been performed in triplicate.

2.7. DNA fragmentation assays

Transfected cells were washed twice with cold PBS and lysed with 2% NP-40 containing 0.2 mg/ml Proteinase K. DNA in the lysate was precipitated with two volumes of ethanol. The pellets were dissolved in H2O and analyzed by electrophoresis with 1.2% gel.

3. Results

3.1. PIASy inhibits TRIF-induced NF-κB activation

Previous reports have shown that TRIF activates NF-κB through TRAF6-IKKβ-dependent pathway when overexpressed in mammalian cells [8,9,13,16]. To examine the role of PIASy in TRIF-mediated NF-κB activation, we performed transient transfection and reporter gene assays in 293 cells. The results indicated that PIASy inhibited TRIF-mediated NF-κB activation in a dose-dependent manner (Fig. 1A). In these experiments, PIASy did not affect TRIF expression level (Fig. 1A). TRIF also inhibited TRAF6-mediated NF-κB activation (Fig. 1B) but not TRAF6-mediated c-Jun activation (Fig. 1C). In fact, TRAF6-mediated c-Jun activation was potentiated by PIASy (Fig. 1C).

3.2. PIASy inhibits TRIF-, IRF-3-, IRF-7 and Sendai virus-induced ISRE activation

Previously, it has been shown that TRIF activates both NF-κB and ISRE [8–16]. In reporter gene assays, we found that PIASy could also inhibit TRIF-, IRF-3- and IRF-7-induced ISRE activation (Figs. 2A and B). In these experiments, PIASy also did not affect the expression levels of IRF-3 and IRF-7 (Fig. 2B).

Infection of target cells with Sendai viruses leads to strong activation of ISRE and type I interferon production [29]. As shown in Fig. 2C, Sendai virus-induced ISRE activation was potently inhibited by PIASy in a dose-dependent manner.

3.3. PIASy inhibits TRIF-induced IFN-β activation

Activation of type I IFNs requires a coordinate activation of multiple transcription factors, including NF-κB and IRF-3/IRF-7 [30,31]. It has been shown that TRIF activates both NF-κB and IRF-3/IRF-7 and is required for TLR3-and TLR4-induced IFN-β activation [8,9]. In reporter gene assays,

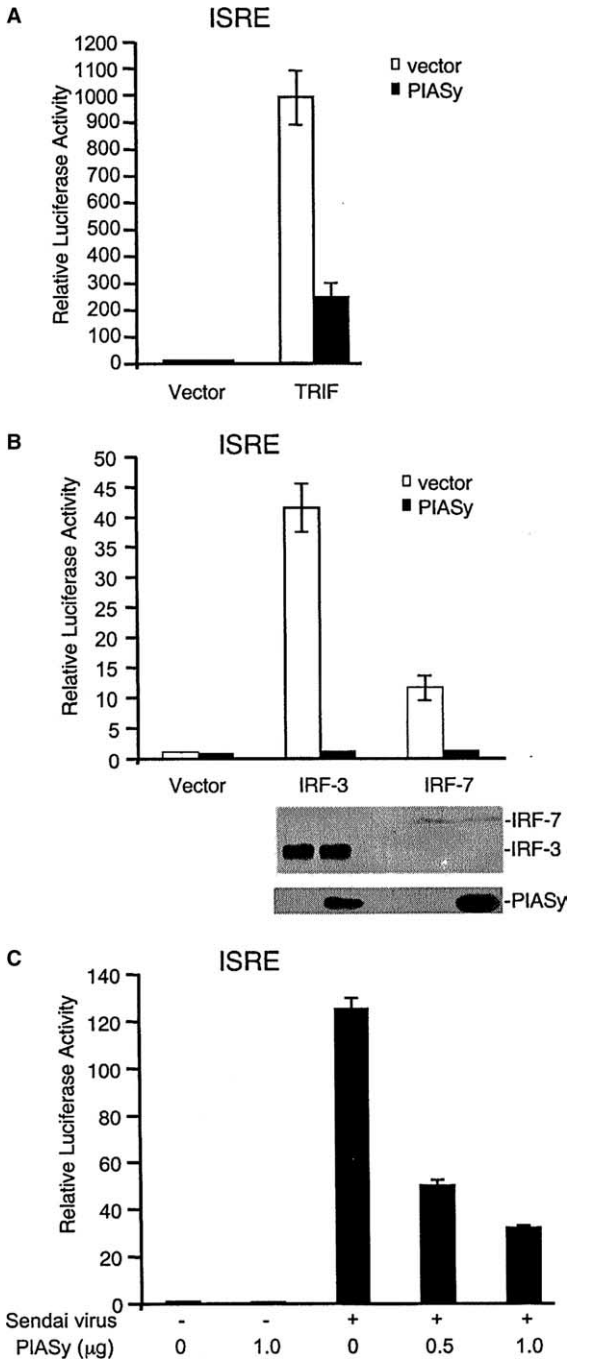

Fig. 2. PIASy inhibits TRIF-, IRF-3-, IRF-7- and Sendai virus-induced ISRE activation. (A) PIASy inhibits TRIF-induced ISRE activation. 293 cells ($\sim 2 \times 10^5$) were transfected with 0.3 μg of ISRE reporter plasmid, 0.3 μg of RSV-β-gal plasmid and 1 μg of each of the indicated expression plasmids. Sixteen hours after transfection, luciferase reporter assays were performed. (B) PIASy inhibits IRF-3- and IRF-7-induced ISRE activation. Reporter assays were performed as in A except that TRIF was replaced with IRF-3 or IRF-7 (upper panel). The expression levels of transfected proteins were detected by Western blot analysis (lower panel). (C) PIASy inhibits Sendai virus-induced ISRE activation. 293 cells ($\sim 1 \times 10^5$) were transfected with 0.3 μg of ISRE reporter plasmid, 0.3 μg of RSV-β-gal plasmid and the indicated amounts of an expression plasmid for PIASy. Eighteen hours after transfection, cells were infected with Sendai virus or left untreated for additional 18 h before luciferase activities were measured.

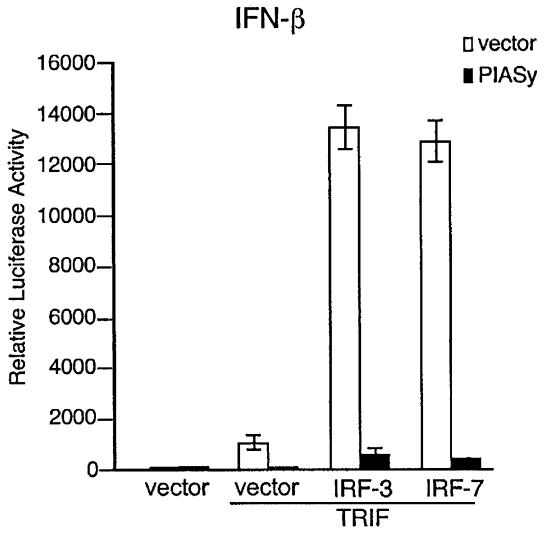

Fig. 3. PIASy inhibits TRIF-induced IFN-β activation. 293 cells ($\sim 2 \times 10^5$) were transfected with 0.3 μg of IFN-p-luciferase reporter construct, 0.3 μg of RSV-β-gal plasmid and the indicated expression plasmids (1 μg each). Sixteen hours after transfection, luciferase reporter assays were performed.

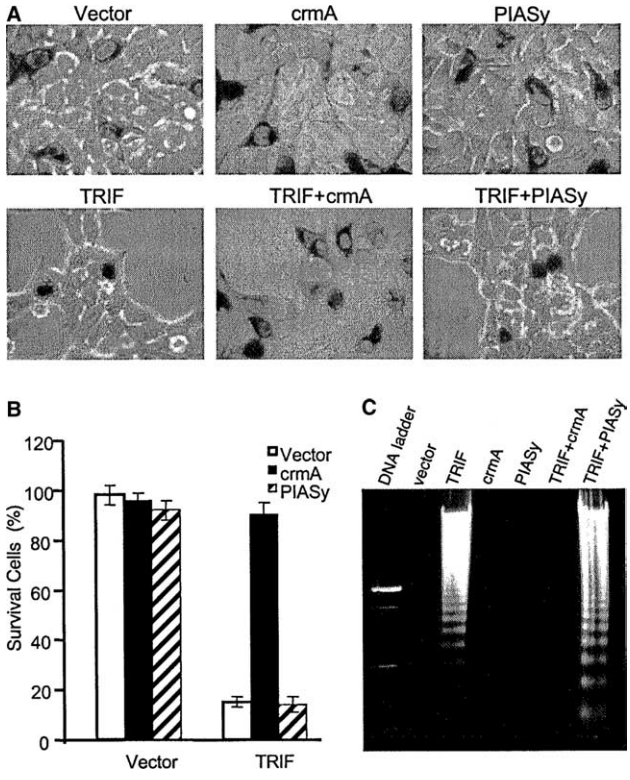

Fig. 4. PIASy has no effects on TRIF-induced apoptosis. (A,B) Effects of crmA and PIASy on TRIF-induced cell death. 293 cells ($\sim 2 \times 10^5$) were transfected with 1 μg of each of the indicated expression plasmids and 0.1 μg of pCMV-β-gal plasmid. Twenty four hours after transfection, the cells were fixed, stained with X-gal and photographed (A). The survived blue cells from five representative viewing fields were quantitated by counting under a microscope (B). (C) Effects of crmA and PIASy on TRIF-induced DMA fragmentation. 293 cells ($\sim 1 \times 10^6$) were transfected with either 10 μg of each of the indicated expression plasmids. DNA was isolated from each sample and analyzed by agarose gel eletrophoresis.

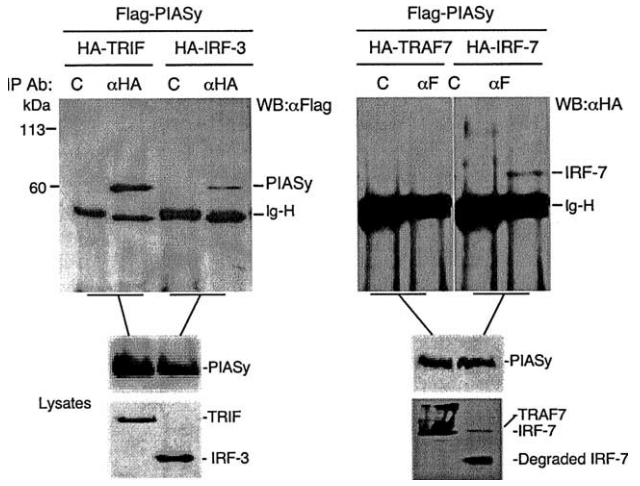

Fig. 5. PIASy interacts with TRIF, IRF-3 and IRF-7 in mammalian cells. 293 cells ($\sim 2 \times 10^6$) were transfected with expression plasmids for Flag-tagged PIASy, together with HA-tagged TRIF, IRF-3, IRF-7 and TRAF7. Cell lysates were immunoprecipitated with control mouse IgG, monoclonal anti-Flag or anti-HA antibody. The immunoprecipitates were analyzed by Western blots with anti-Flag or anti-HA antibody, respectively (upper panels). The expression levels of the transfected proteins were detected by Western blot analysis (middle and bottom panels).

we found that overexpression of PIASy could dramatically inhibit TRIF-induced activation of IFN-β promoter (Fig. 3).

3.4. PIASy has no effects on TRIF-induced apoptosis

Infection of cells with Sendai viruses induces apoptosis [32]. It has been demonstrated that TRIF induces caspase-dependent apoptosis when overexpressed in 293 cells [16]. To determine whether PIASy is involved in TRIF-induced apoptosis, we performed β-galactosidase co-transfection and DNA fragmentation assays. In these assays, TRIF-induced apoptosis was inhibited by the caspase inhibitor crmA but not by PIASy (Figs. 4A–C).

3.5. PIASy interacts with TRIF, IRF-3 and IRF-7 in mammalian cells

PIAS family members exert their biological effects by directly interacting with multiple molecules [17–23]. Since PIASy can inhibit TRIF-induced NF-κB, ISRE and IFN-β activation, we determined whether PIASy could interact with TRIF, IRF-3 and IRF-7 in mammalian cells. We transfected 293 cells with an expression vector for Flag-tagged PIASy together with an expression vector for HA-tagged TRIF, IRF-3 or IRF-7. Co-immunoprecipitation and Western blot experiments indicated that PIASy could interact with TRIF, IRF-3 and IRF-7 (Fig. 5). In contrast, a control protein, TRAF7, did not interact with PIASy in these experiments (Fig. 5). These data suggest that PIASy interacts with TRIF, IRF-3 and IRF-7.

4. Discussion

The TIR family members of the innate immune system detect host invasion by pathogens and initiate immune responses. All of the TIR receptors use the adaptor MyD88 to transduce a signal; however, three newly identified signaling molecules,

TIRAP, TRIF and TIRP, interact with a subset of the TLRs, suggesting a signaling specificity that may be relevant to the type of infection [33].

TLR3 recognizes dsRNA generated during viral infection and is critically involved in host defense against viruses [34]. Activation of TLR results in induction of type I IFNs, including IFN-β and IFN-α family cytokines, which are crucial mediators of the anti-viral response. TRIF is the TIR-domain containing adapter protein critically involved in TLR3-induced IFN-β production and NF-κB activation [8,9].

Previously, we and others have shown that TRIF activates NF-κB and ISRE and induces apoptosis through distinct pathways [8–16]. In this study, we found that PIASy could inhibit TRIF-induced NF-κB and ISRE activation but not apoptosis. Interestingly, PIASy could also inhibit NF-κB activation induced by TRAF6, a downstream component of TRIF-induced NF-κB activation pathway. One explanation for this is that PIASy also targets TRAF6 or a component downstream of TRAF6.

Previously, it has been reported that PIASy is located predominantly in the nucleus and interacts with various transcription factors [23,26,27,35]. However, it has also been reported that PIASy interacts with cytoplasmic proteins, such as Axin [36]. It has been shown that TRIF is associated with Toll-like receptor 3 [8,9]. Whether TRIF is transported into the nucleus is unknown. IRF-3 and IRF-7 are normally located in the cytoplasm, but are transported into the nucleus upon activation [8,9]. Currently, where PIASy interacts with TRIF, IRF-3 and IRF-7 are unknown.

Previous studies have shown that PIASy can repress transcriptional activities through different mechanisms. PIASy inhibits STAT1 and androgen receptor activities without interfering with their DNA-binding activities, whereas the repression of p53 activity appears to be through inhibition of p53 binding to DNA [23,26,35]. On another hand, it has been shown that the inhibitory effect of LEF1 by PIASy is mediated by sequestration of LEF1 into nuclear bodies [27]. The mechanisms responsible for PIASy-mediated inhibition of TRIF-induced NF-κB and ISRE activation are unknown. Since PIASy has been shown to function as a SUMO ligase, whether sumoylation is involved in PIASy-mediated inhibition of TRIF signaling needs to be further investigated.

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