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Interaction of IFNλR1 With TRAF6 Regulates NF-κB Activation and IFNλR1 Stability

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ABSTRACT

IFNλR1 is a member of the class II cytokine receptor family, and it associates with IL-10R2 to form a functional receptor complex, IFNλR. This receptor complex transduces signals from IFNλs (IFNλ1, IFNλ2, and IFNλ3), promoting antiviral and antiproliferative activities similar to those of type I IFNs. In an effort to further understand signal transduction through IFNλR1, we used bioinformatics analysis and identified a tumor necrosis factor receptor-associated factor 6 (TRAF6)-binding motif in the intracellular domain of IFNλR1. In subsequent immunoprecipitation and GST pull-down assays, IFNλR1 was shown to immunoprecipitate with TRAF6 and was pulled down by GST-TRAF6. Endogenous IFNλR1 and TRAF-6 interaction implies that these proteins really interact in the cells. This interaction was abrogated upon mutation of the TRAF6-binding motif in IFNλR1. Furthermore, the interaction between IFNλR1 and TRAF6 inhibited TRAF6-induced NF-κB activation, likely due to a reduction in TRAF6 autoubiquitination. Moreover, co-expression of IFNλR1 with TRAF6 significantly increased the stability of IFNλR1, thereby prolonging its half-life and enhancing its steady-state level in cultured cells. J. Cell. Biochem. 113: 3371–3379, 2012.

KEY WORDS: IFNλR1; TRAF6; INTERACTION; NF-κB; PROTEIN STABILITY

Interferon (IFN)λs are newly identified IFN-like cytokines. The IFNλ family includes IFNλ1, IFNλ2, and IFNλ3 (which are also named IL-29, IL-28A, and IL-28B, respectively). IFNλs represent an interesting link between the IL-10 family and type I IFNs (IFNα/β). Functionally, they display antiviral and antiproliferative activities similar to those of type I IFNs [Kotenko et al., 2003; Sheppard et al., 2003; Zitzmann et al., 2006; Pagliaccetti et al., 2010; Witte et al., 2010]. However, IFNλ and IFNα differ in some functions, such as their ability to modulate IL-12 production by TLR-activated human macrophages and to regulate IFNγ receptor expression [Liu et al., 2011]. The IFNλ receptor is a member of the class II cytokine receptor family (CRF2), which includes receptors for type I and type II IFNs (IFNAR1, IFNAR2, IFNGR1, and IFNGR2), IL-10R (IL-10R1 and IL-10R2/CRF2-4) and receptors for IL-10 related cytokines (IL-20R1/CRF2-8, IL-20R2/CRF2-11, IL-22R1/CRF2-9, and IFNλR1/IL-28Rα/CRF2-12) [Dumoutier and Renauld, 2002; Langer et al., 2004]. All of the class II cytokine receptor complexes consist of two subunits, R1 and R2. IFNλR1 dimerizes with IL-10R2 to form a functional IFNλR complex, forming a unique ligand-binding chain. The crystal structure of human IFNλ1 in complex with its high-affinity receptor, IFNλR1, proved that long-range ionic interactions between the ligand and receptor govern the process of initial recognition of the molecules, whereas hydrophobic interactions finalize it [Miknis et al., 2010]. IFNλR1 also has a long intracellular domain that can be phosphorylated on tyrosine residues, determining the specificity of cytokine signaling

Yun-Fei Xie and Ying-Bin Cui contributed equally to this work.

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[Dumoutier et al., 2004]. IL-10R2 has a short intracellular domain and supports signaling by recruiting an additional tyrosine kinase. Although they use distinct receptor chains, which have no detectable homology in their intra-cytoplasmic domain, IFNλs and type I IFNs both activate the Jak-STAT (Janus kinases-signal transducers and activators of transcription) pathway [Kotenko et al., 2003; Dumoutier et al., 2004; Guenterberg et al., 2010]. Interestingly, in contrast to IFNα, IFNλ signals enhance STAT1 and STAT2 tyrosine phosphorylation and lead to prolonged ISG (IFN-stimulated regulatory factor) expression [Maher et al., 2008]. However, knowledge concerning IFNλ and IFNλR1 is still limited, and further insight into the mechanisms underlying the signaling of IFNλR is needed.

Mammalian TNF receptor-associated factors (TRAFs) constitute a seven-member family of conserved cytoplasmic adapter proteins that can interact directly with the intracellular domains of cell surface receptors. TRAFs initiate the assembly of multiprotein complexes that induce downstream events, such as translocation of the transcription factor nuclear factor κB (NF-κB) and activation of c-Jun N-terminal kinase (JNK) [Xu et al., 2004; Yang et al., 2008; Wang et al., 2011]. TRAF6 is the only TRAF family member that has a crucial role as a signaling mediator for both the TNF receptor superfamily and the IL-1R/Toll-like receptor (TLR) superfamily [Chung et al., 2002; Ye et al., 2002; Carpenter and O'Neill, 2009]. Thus, TRAF6 represents a molecular bridge for innate immunity, adaptive immunity, and bone homeostasis [Wu and Arron, 2003; Chung et al., 2007; Xu et al., 2009].

Using bioinformatics method, we found that the sequence of the intracellular domain of IFNλR1 contains a putative TRAF6-binding motif. In the present study, we examined the interaction of IFNλR1 with TRAF6. First, we found that IFNλR1-ICD (the intracellular domain of IFNλR1) and TRAF6 could directly bind in GST pull-down assays and they can co-immunoprecipitate when both exogenously expressed in HEK293T cells. Furthermore, this interaction was abrogated when the TRAF6-binding motif in IFNλR1 was mutated. The interaction between full-length IFNλR1 and TRAF6 in vivo was further confirmed by both semi-endogenous and endogenous immunoprecipitation experiments. We also determined that the interaction between IFNλR1 and TRAF6 inhibits TRAF6-induced NF-κB activation and that this inhibition is likely caused by the reduction in TRAF6 autoubiquitination. Co-expression of IFNλR1 with TRAF6 significantly increased the stability of IFNλR1, thereby prolonging its half-life and enhancing its steady-state level in cultured cells. These results suggest that TRAF6 interacts with IFNλR1 to regulate NF-κB activation and IFNλR1 stability.

MATERIALS AND METHODS

PLASMID CONSTRUCTION

The human IFNλR1-ICD (amino acids 250–520) coding sequence was amplified from a human spleen cDNA library (Clontech) by PCR using the primers ICD-F and λR1-R. The mature IFNλR1 (amino acids 21–250) coding sequence was directly amplified from pEFPL3-IFNλR1 (a generous gift from Dr. Jean-Christophe Renauld, Universite catholique de Louvain, Belgium) by PCR using the primers λR1-F and λR1-R. The two amplified fragments were subcloned in-frame into the *Bgl*II–*Kpn*I sites of pCMV-Myc, resulting in the following constructs: pCMV-Myc-IFNλR1-ICD and pCMV-Myc-IFNλR1. An overlapping PCR technique was used to create the P480A/E482Q-IFNλR1 double mutant with pCMV-Myc-IFNλR1-ICD as a template. The following primer pairs were used to make point mutations: (i) primer ICD-F and ICDM-R and (ii) ICDM-F and primer λR1-R. The mutated fragment was also subcloned at the *Bgl*II–*Kpn*I sites of pCMV-Myc to generate pCMV-Myc-IFNλR1-ICDM. Using the similar method with that of pCMV-Myc-IFNλR1-ICDM, the full-length IFNλR1 mutant expression vector, pCMV-Myc-IFNλR1M, was constructed. Full-length TRAF6 cDNA was PCR-amplified from a human leukocyte cDNA library (Clontech) using the following primers: TRAF6-F1 and TRAF6-R1. The resultant TRAF6 fragment was digested with *Eco*RI and subcloned into the pGEX-6P-1 expression vector, which digested with *Not*I, filled in with Klenow fragment and then digested again with *Eco*RI, to produce pGEX-6P-TRAF6. pFLAG-TRAF6 was a generous gift from Dr. Bruce D. Carter (Vanderbilt University, USA). Using pFLAG-TRAF6 as a template, FLAG-tagged TRAF6 was PCR-amplified with TRAF6-F2 and TRAF6-R2 primers. The FLAG-TRAF6 fragment was digested with *Bgl*II and subcloned into the pBudCE4 vector which was digested with *Bgl*II, after digestion with *Not*I and treatment with the Klenow fragment, to produce pBudCE4-TRAF6. The full-length TRAF2 expression vector, pCMV-HA-TRAF2, was constructed by PCR amplification from a human leukocyte cDNA library using the primers TRAF2-F and TRAF2-R, and then by the PCR fragment in-frame subcloned into the *Bgl*II–*Not*I sites of pCMV-HA. Construction of pCMV-HA-TRAF6 and pEF-BOS-Ub (containing a FLAG-tag) has been described previously [Wang et al., 2011]. The sequences of the primers needed for above mentioned PCR reactions are listed in Table I and all of the constructs were confirmed by DNA sequencing.

CELL CULTURE AND TRANSFECTION

HEK293T cells were cultured in Dulbecco's modified eagle's medium (DMEM; Hyclone) supplemented with 2 mM L-glutamine, 4.5 g/L glucose and 10% fetal bovine serum. Raji cells were grown in RPMI-1640 (Hyclone) medium with 2 mM L-glutamine and 10% fetal bovine serum. Cells were transfected with transfection reagents (Vigorous) according to the manufacturer's protocol.

TABLE I. Primer Name and Sequence

Primer name	Primer sequence
ICD-F	5′-GAAGATCTTGAAGACCCTCATGGGGAAC-3′
λR1-R	5′-GCGGTACCTCACCTGGCCATGTAATGC-3′
λR1-F	5′-GCAGATCTCTCCAGGGAGGCCCCGTCTGGC-3′
ICDM-R	5′-GCTGCTTTCCCAGCAGAAG-3′
ICDM-F	5′-CTGCTGGGAAAGCAGCGCTGAGCAGGAAGAGGAGGCGAG-3′
ICDM-RN	5′-CTTCCTGCTCAGCGCTGCTTTCCCAGCAGAAG-3′
TRAF6-F1	5′-CGGAATTCACTATGAGTCTGCTAAACTGTG-3′
TRAF6-R1	5′-GCTATACCCCTGCATCAGTACT-3′
TRAF6-F2	5′-GCGCCGCCACCATGGACTACAAGGAC-3′
TRAF6-R2	5′-GAAGATCTATACCCCTGCATCAGTACT-3′
TRAF2-F	5′-TAAGATCTTCATGGCTGCAGCTAGCGTGAC-3′
TRAF2-R	5′-TTGCGGCCGCGTTAGAGCCCTGTCAGGTCCAC-3′

CO-IMMUNOPRECIPITATION AND WESTERN BLOT

Transfected HEK293T cells were harvested 48 h post-transfection and lysed in 1 ml RIPA lysis buffer containing a protease inhibitor cocktail (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin). Immunoprecipitations were performed using equal amounts of lysate and a rabbit anti-Myc polyclonal antibody (Santa Cruz) or a mouse anti-FLAG monoclonal antibody (Sigma) overnight at 4°C. Subsequently, protein-A sepharose beads were added, and the incubation was continued for another 2 h at room temperature. After washing the beads three times with NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin), the immunocomplexes were eluted by boiling in 50 μl 1× SDS sample buffer for 5 min. Proteins were separated on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose (NC) membrane. Protein blots were blocked with 5% non-fat milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20), and incubated with anti-FLAG, anti-Myc, or anti-TRAF6 (Santa Cruz) antibody for 2 h at 4°C. The membrane was washed with TBST and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit or anti-mouse IgG (Santa Cruz). The signal was detected using Super Signal chemiluminescence (Thermo Scientific Pierce) and exposed to X-ray film.

GLUTATHIONE *S*-TRANSFERASE (GST) PULL-DOWN ASSAY

A GST-TRAF6 fusion protein was generated by induction of *Escherichia coli* BL21/pGEX-6P-TRAF6 in LB media containing 0.5 mM IPTG and 10 mM $ZnCl_2$ overnight at room temperature. The bacterial pellets were resuspended in STE buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 μg/ml lysozyme, 3% Triton X-100), and lysed by sonication. The cleared lysates were then incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia) for 2 h at room temperature and after the BL21 lysates were removed out by centrifugation, the beads were incubated with the lysates of HEK293T cell transfected with pCMV-Myc-IFNλR1-ICD overnight at 4°C. Next, the beads were centrifuged and washed with cold phosphate-buffered saline (PBS) three times and resuspended in 1× SDS-loading buffer. Proteins were separated by 8% SDS–polyacrylamide gel and transferred to a NC membrane. The blots were probed with antibodies directed against Myc.

IMMUNOPRECIPITATION OF ENDOGENOUS IFNλR1 AND TRAF-6

Raji cells were cultured in 90 mm dishes and treated with 200 ng/ml IFNλ1 [Xie et al., 2007]. The cells were harvested 5- or 60-min post-stimulation and lysed in RIPA lysis buffer containing a protease inhibitor cocktail. Lysates were then immunoprecipitated using the anti-TRAF6 antibody, the immune complexes were separated by SDS–PAGE, and Western blotting was used to detect IFNλR1 and TRAF6.

LUCIFERASE REPORTER ASSAY

HeLa cells were grown in 24-well plates in DMEM and transfected with 0.202 μg plasmid, including 0.2 μg pNF-κB-Luc, 0.002 μg pRL-SV40 (Promega, which expresses the *Renilla* luciferase gene and was used as an internal control), plus 1 μg of either pCMV-Myc-IFNλR1 or pBudCE4-TRAF6, or together with them. Four hours post-transfection, HeLa cells were treated with 200 ng/ml IFNλ1 for 20 h, and cells were harvested and lysed using Promega 1× passive lysis buffer. Relative NF-κB-Luciferase activity was measured using the dual luciferase reporter assay system (Promega). The mix of cell lysates and buffer was tested on a SPECTRA MAX XPS (Molecular Devices).

RESULTS

IFNλR1-ICD SPECIFICALLY INTERACTS WITH TRAF6

The TRAF6-binding motif is a consensus Pro-X-Glu-X-X (Ar/Ac) sequence [Ye et al., 2002]. Bioinformatic analysis of the amino acid sequence of IFNλR1 indicated a putative TRAF6-binding motif at amino acid position 480–485, consisting of Pro-Glu-Glu-Glu-Glu-Glu (PEEEEE; http://blast.ncbi.nlm.nih.gov/Blast.cgi). To verify that IFNλR1 is able to interact with TRAF6, we examined whether IFNλR1 could be co-immunoprecipitated with TRAF6. HEK293T cell cultures were co-transfected with expression constructs that directed synthesis of FLAG-tagged TRAF6 and Myc-tagged IFNλR1-ICD. The molecular weight (MW) of Myc-IFNλR1-ICD is 33 kDa, as deduced from its amino acid composition, whereas Myc-IFNλR1-ICD expressed in HEK293T cells comprises two forms with a MW of approximately 42 and 45 kDa (Fig. 1A), suggesting that the protein may be post-translationally modified. Cell lysates prepared from these co-transfected cells were then immunoprecipitated with anti-FLAG monoclonal antibody or anti-Myc polyclonal antibodies, followed by Western blotting with anti-Myc or anti-FLAG antibodies, respectively. Figure 1B shows that the IFNλR1-ICD protein co-immunoprecipitated with TRAF6 when pulled down by anti-FLAG antibody, and TRAF6 co-immunoprecipitated with IFNλR1-ICD when pulled down by anti-Myc antibody. Western blot analysis of co-immunoprecipitates revealed that only the 42 kDa band of the IFNλR1-ICD protein interacts with TRAF6. Co-immunoprecipitation assays were also performed using IFNλR1-ICD and TRAF2, but no interaction was observed (data not shown).

The interaction between TRAF6 and IFNλR1 was also tested by GST pull-down assay. Full-length TRAF6 fused to a GST-tag was expressed in BL21 (DE3), and the recombinant fusion protein was incubated with the lysates of HEK293T cells transfected with Myc-IFNλR1-ICD (Fig. 1C). The GST-bound material was separated by SDS–PAGE, and then Western blotting was used to detect IFNλR1-ICD with anti-Myc antibody. As shown in Figure 1D, the TRAF6-GST fusion protein, but not the GST protein, bound IFNλR1-ICD prepared from the HEK293T cells expressing Myc-IFNλR1-ICD. Consistent with the co-immunoprecipitation assay, only the 42 kDa IFNλR1-ICD could be pulled down by GST-TRAF6. Taken together, both the in vivo and in vitro assays support that finding that IFNλR1-ICD directly interacts with TRAF6.

THE INTERACTION BETWEEN IFNλR1 AND TRAF6 REQUIRES THE TRAF6-BINDING MOTIF (P480 AND E482)

Several proteins known to interact with TRAF6 have a conserved TRAF6-binding motif (Fig. 2A), and disruption of the TRAF6-binding motif blocked their association with TRAF6 [Jiang et al., 2004; Mansell et al., 2004]. To further determine whether the

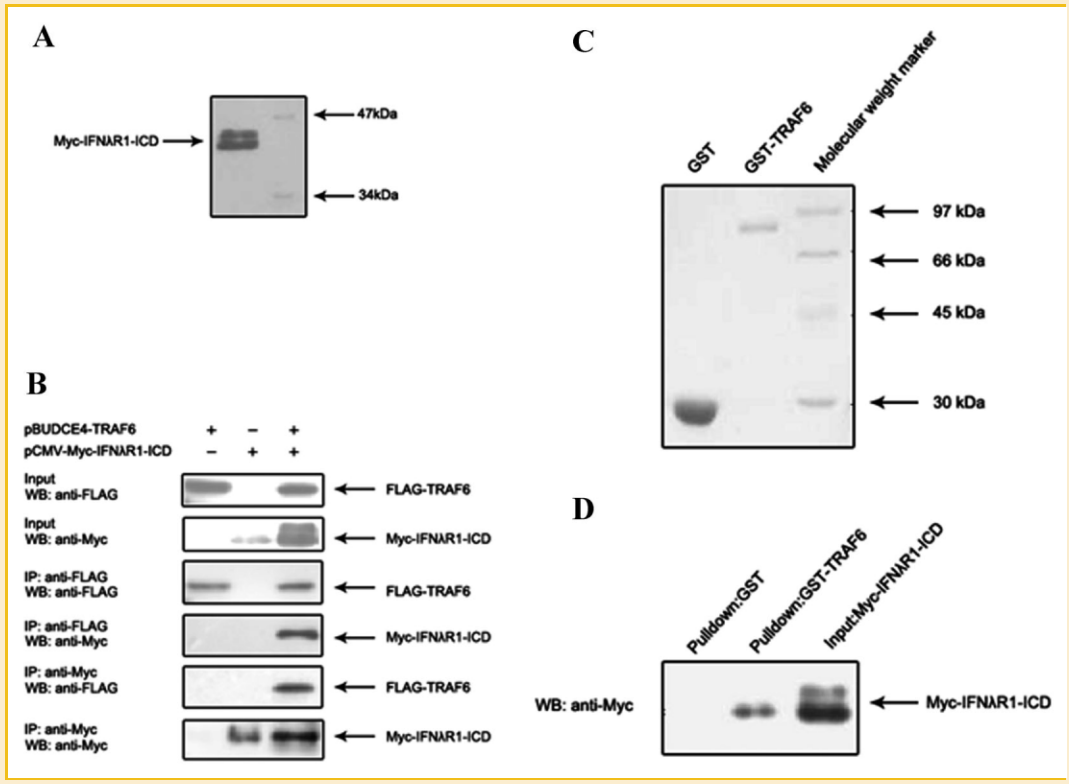

Fig. 1. The interaction between IFNλR1-ICD and TRAF6. A: IFNλR1-ICD (intracellular domain) was expressed as two glycoproteins in HEK293T cells transfected with Myc-tagged IFNλR1-ICD. The cell lysates were separated by SDS–PAGE, and then Western blotting was performed with anti-Myc antibody. Myc-IFNλR1-ICD comprised of two bands with an apparent MW of approximately 42 and 45 kDa. B: IFNλR1 and TRAF6 interact in cells. HEK293T cell were transfected with pCMV-Myc-IFNλR1-ICD or pBudCE4-TRAF6 alone or were co-transfected with both plasmids. Forty-eight hours after transfection, the cells were lysed, and IFNλR1-ICD and TRAF6 were immunoprecipitated with anti-Myc and anti-FLAG antibodies, respectively. The Myc and FLAG immunoprecipitate complexes were resolved by SDS–PAGE, and Western blotting with antibodies specific for FLAG or Myc to detect TRAF6 or IFNλR1-ICD. C: SDS–PAGE of GST and GST-TRAF6 bound to glutathione sepharose 4B beads. *Escherichia coli* were transformed with pGEX-6P-1 or pGEX-6P-TRAF6 and induced with IPTG to produce GST and GST-TRAF6 proteins. *E. coli* lysates were incubated with glutathione sepharose 4B beads. The binded proteins were separated by 10% SDS–PAGE, and the gel was stained with Coomassie brilliant blue. D: GST pull-down assay. GST-TRAF6 bound to GST beads was incubated with the lysates of the HEK293 cells transfected with Myc-IFNλR1-ICD overnight at 4°C. The beads were washed with cold PBS three times and resuspended in 1× SDS-loading buffer. The bound proteins were analyzed by 10% SDS–PAGE, and Western blotting was performed with anti-Myc antibody. IFNλR1-ICD was found in the GST-TRAF6 lane (middle) but not in the GST lane (left). The right lane is a positive input control.

TRAF6-binding motif in IFNλR1 was involved in the association with TRAF6, we mutated this motif by replacing the proline at position 480 with alanine (P480A) and the glutamic acid at position 482 with glutamine (E482Q) and tested the ability of the mutated protein to interact with TRAF6 by co-immunoprecipitation analysis. As shown in Figure 2B, the mutated form of IFNλR1-ICD was unable to interact with TRAF6. The similar result was observed in the interaction between IFNλR1M and TRAF6, which was shown in Supplementary Figure S1B. These results show a genuine TRAF6-binding site in IFNλR1 that contributes to the association between IFNλR1 and TRAF6.

IFNλ1 REDUCES THE INTERACTION BETWEEN ENDOGENOUS IFNλR1 AND TRAF6

Next, we explored whether endogenous IFNλR1 and TRAF6 interact and whether endogenous IFNλR1 interacts with TRAF6 in response to ligand (IFNλ1) stimulation. We first conducted semi-endogenous co-immunoprecipitation experiments. The lysates of HEK293T cells expressing pCMV-Myc-IFNλR1 or pCMV-Myc were subjected to immunoprecipitation with anti-Myc antibody, followed by Western blotting with the anti-TRAF6 antibody (Santa Cruz). As shown in Figure 3A, overexpressed IFNλR1 interacted with endogenous TRAF6. To further verify an endogenous interaction between TRAF6 and IFNλR1, Raji cell were used for endogenous immunoprecipitation assays. TRAF6 was immunoprecipitated from Raji cell lysates with anti-TRAF6 antibody, and Western blotting revealed a clear interaction with endogenous IFNλR1 in this immune complex (Fig. 3B). At the same time, we examined whether endogenous IFNλR1 could interact with TRAF6 in response to ligand (IFNλ1) stimulation. Raji cells were treated with 200 ng/ml IFNλ1. Surprisingly, the association between TRAF6 and IFNλR1 was markedly reduced 5 min after IFNλ1 stimulation (Fig. 3B). A similar result was observed in HL-60 cells and HeLa cells (data not shown).

IFNλR1 INHIBITS TRAF6 INDUCED-NF-κB ACTIVATION

To further explore the function of the IFNλR1-TRAF6 association on signaling pathway activation, we conducted luciferase reporter assays and in vivo ubiquitination assays. HeLa cells were transfected

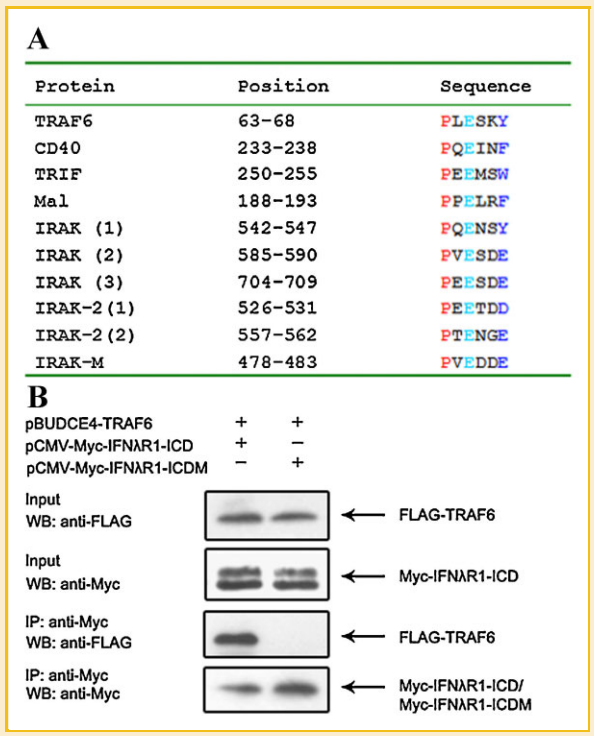

Fig. 2. The TRAF6-binding motif of IFNλR1 is required for its interaction with TRAF6. A: List of proteins containing a TRAF6-binding motif: Pro-X-Glu-X-X(Ar/Ac) [Ye et al., 2002; Mansell et al., 2004]. B: HEK293T cells were co-transfected with pBudCE4-TRAF6 and pCMV-Myc-IFNλR1-ICD or with pBudCE4-TRAF6 and the mutant IFNλR1-ICD-carrying vector pCMV-Myc-IFNλR1-ICDM (P480A, E482Q). Forty-eight hours after transfection, the cell lysates were immunoprecipitated with anti-Myc antibody, and the precipitates were resolved by SDS–PAGE and immunoblotted with antibodies against FLAG. TRAF6 was found to interact with IFNλR1-ICD but not with IFNλR1-ICDM.

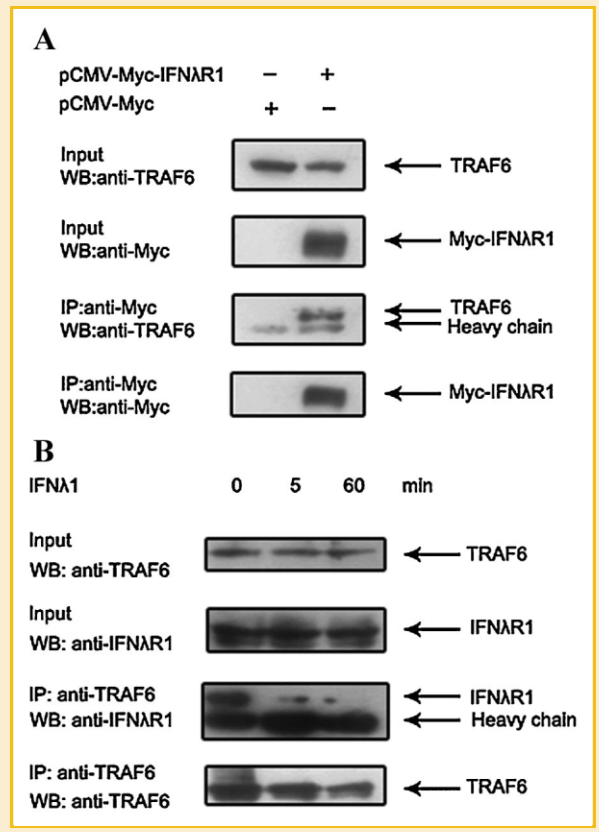

Fig. 3. Endogenous interaction between IFNλR1 and TRAF6. A: Semiendogenous interaction between overexpressed IFNλR1 and endogenous TRAF6. HEK293T cells were transfected with the expression plasmid pCMV-Myc-IFNλR1 or pCMV-Myc. Forty-eight hours after transfection, the cells were lysed, and IFNλR1 was immunoprecipitated with anti-Myc antibody. The Myc immunoprecipitates were resolved by SDS–PAGE, and Western blotting was performed with anti-TRAF6 or anti-Myc antibodies. B: The interaction between endogenous IFNλR1 and endogenous TRAF6. Raji cells were treated with or without IFNλ1, 200 ng/ml for 5 or 60 min, then lysed and immunoprecipitated with anti-TRAF6 antibodies. Cell lysates and immunoprecipitates were separated by SDS–PAGE, and immunoblotting was used to detect Endogenous IFNλR1 and Endogenous TRAF6.

with pCMV-Myc-IFNλR1 or pBudCE4-TRAF6 alone or were co-transfected with both of the plasmids in the presence of pNF-κB-Luc and pRL-SV40. As shown in Figure 4A, overexpression of TRAF6 resulted in dramatic stimulation of NF-κB reporter activity. However, when stimulated with IFNλ1, TRAF6 induced-NF-κB activation was largely inhibited by co-expression of IFNλR1, in a dose-dependent manner (Fig. 4B). Further, we found that the inhibition was reduced when the TRAF6-binding motif was disrupted (Supplementary Fig. S1B).

In ubiquitination assays, HEK293T cells were transfected with pCMV-Myc-IFNλR1, pCMV-HA-TRAF6, and pEF-BOS-Ub. Forty-eight hours after transfection, cells were harvested and lysed. After immunoprecipitation with the anti-HA antibody, the precipitates were resolved by SDS–PAGE and subjected to immunoblotting with anti-FLAG or anti-TRAF6 antibodies. As can be seen in Figure 4C, IFNλR1 activation reduced the autoubiquitination of TRAF6.

TRAF6 INCREASES THE STABILITY OF IFNλR1

In co-immunoprecipitation assays, we found that the amount of Myc-IFNλR1-ICD was higher in the lysates of cells co-transfected with Myc-IFNλR1-ICD and FLAG-TRAF6 than in those expressing Myc-IFNλR1-ICD only. Thus, we hypothesized that the interaction between TRAF6 and IFNλR1 affects the steady-state level of IFNλR1. To test whether the level of IFNλR1 was affected by TRAF6, HEK293T cells were co-transfected with a constant amount of pCMV-Myc-IFNλR1, in the presence or absence of increasing amounts of pBudCE4-TRAF6. As can be seen in Figure 5A, Myc-IFNλR1 expression levels increased in a TRAF6 dose-dependent manner in the presence of FLAG-TRAF6, whereas HA-TRAF2 had no significant effect on Myc-IFNλR1 levels (Fig. 5B).

We next sought to determine whether the half-life of IFNλR1 differed in the presence of TRAF6. HEK293T cells were co-transfected with a constant amount of pCMV-Myc-IFNλR1, with or without pBudCE4-TRAF6, and treated with cycloheximide to inhibit protein synthesis 48 h after transfection. At the indicated time points, cell lysates were collected, and IFNλR1 levels were monitored by Western blotting. As shown in Figure 5C, IFNλR1 had a short

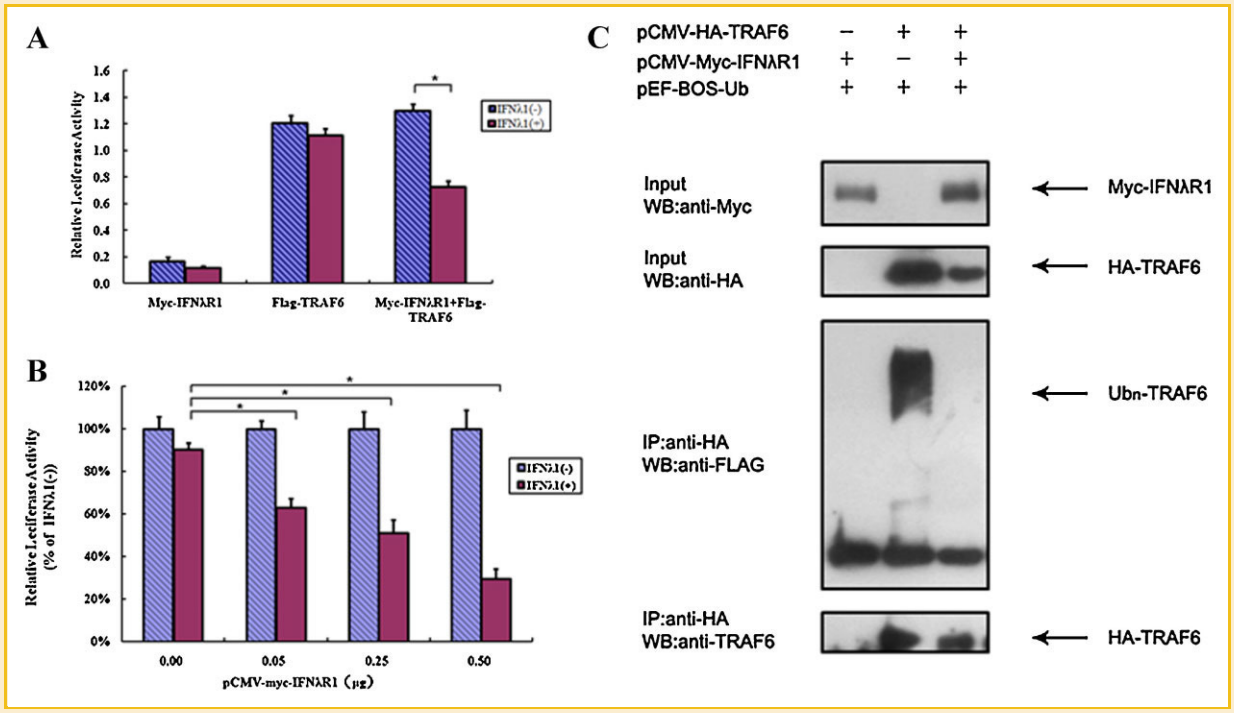

Fig. 4. IFNλ inhibits TRAF6-mediated activation of NF-κB and reduces auto ubiquitination of TRAF6. A: HeLa cells were transfected with pCMV-Myc-IFNλR1 or pBudCE4-TRAF6 alone or were co-transfected with both of these plasmids in the presence of pNF-κB-Luc and pRL-SV40. Four hours later, cells were stimulated with or without IFNλ1 (200 ng/ml for 20 h). Luciferase activities were determined by a Dual-Luciferase Reporter Assay System. Results represent the mean ± SD. $^{*}P < 0.01$. B: HeLa cells were co-transfected with pBudCE4-TRAF6 and different amounts of pCMV-Myc-IFNλR1 (0–0.5 μg) in the presence of pNF-κB-Luc and pRL-SV40. Four hours later, the cells were stimulated with or without IFNλ1, 200 ng/ml for 20 h. Luciferase activities were determined by a Dual-Luciferase Reporter Assay System. Results represent the mean ± SD. $^{*}P < 0.01$. C: HEK293T cells were transfected with pCMV-Myc-IFNλR1 or pCMV-HA-TRAF6 alone or were co-transfected with both of these plasmids in the presence of pEF-BOS-Ub. Forty-eight hours post-transfection, the cells were harvested and lysed. The cell lysates were immunoprecipitated with anti-HA antibody and resolved by SDS–PAGE, and immunoblotting was used to detect FLAG or TRAF6.

half-life of <4 h, whereas co-expression of FLAG-TRAF6 and IFNλR1 dramatically decreased the rate of IFNλR1 degradation, resulting in a half-life of more than 8 h (Fig. 5D). Therefore, TRAF6-dependent stabilization of IFNλR1 is attributed to the elongation of the half-life of IFNλR1, leading to an increase in the amount of IFNλR1.

DISCUSSION

The discovery and initial description of the interferon-λ (IFNλ) family in 2003 opened an exciting new chapter in the field of IFN research. Interferon-λ is functionally an interferon but is structurally related to the interleukin-10 family [Gad et al., 2009]. IFNλ signals through the IFNλR, activating the JAK-STAT and MAPK pathways and thereby inducing antiviral, antiproliferative, antitumor, and immune responses [Li et al., 2009]. The potential clinical importance of IFNλ is already apparent, and it may also be useful as a therapeutic agent for cancer and viral infection. For example, it may provide a promising treatment strategy for HCV patients, because the IFNλ1 levels of patients with chronic hepatitis C are substantially lower than those of healthy controls ($P = 0.005$) and patients with spontaneously resolved hepatitis ($P = 0.001$) [Langhans et al., 2011]. IFNλ are distinct from both type I and type II IFNs for a number of reasons, including because they signal through a heterodimeric receptor complex that is different from those used by type I or type II IFNs [Donnelly and Kotenko, 2010]. Class II cytokine receptors are heterodimers, except for tissue factor (TF). Within each heterodimeric receptor, the intracellular domain of one subunit (R1) is considerably larger than that of its partner (R2), and R1 also has a substantially higher affinity for its ligand than R2 [Langer et al., 2004]. IFNλR is composed of IFNλR1 and IL-10R2. Thus, the R1 subunit of IFNλR, IFNλR1, determines the specificity of IFNλ signaling. IL-10R2 also functions as a receptor subunit in the IL-10, IL-22, and IL-26 receptor complexes. Based on our knowledge of IL-10, IL-22, and IL-26, the function of IL-10R2 in signaling is limited to recruitment of the tyrosine kinase Tyk2. Downstream signaling events, such as inducing the phosphorylation of Jak1 and STATs, are not dependent on IL-10R2 but rather on the larger subunits, IL-10R1, IL-22R1, and IL-26R1 [Lejeune et al., 2002; Sheikh et al., 2004; Zdanov, 2010]. As a member of the CRF2, IFNλR1 has functional similarities to both IFNAR2 and IL-10R1. However, knowledge concerning IFNλR1 is still limited.

To gain further insight into the mechanisms underlying IFNλR signaling, it is necessary to identify which cellular proteins can associate with IFNλR1. Scanning of the IFNλR1 amino acid sequence revealed a consensus TRAF6-binding motif in the intracellular domain. It has been reported that many proteins,

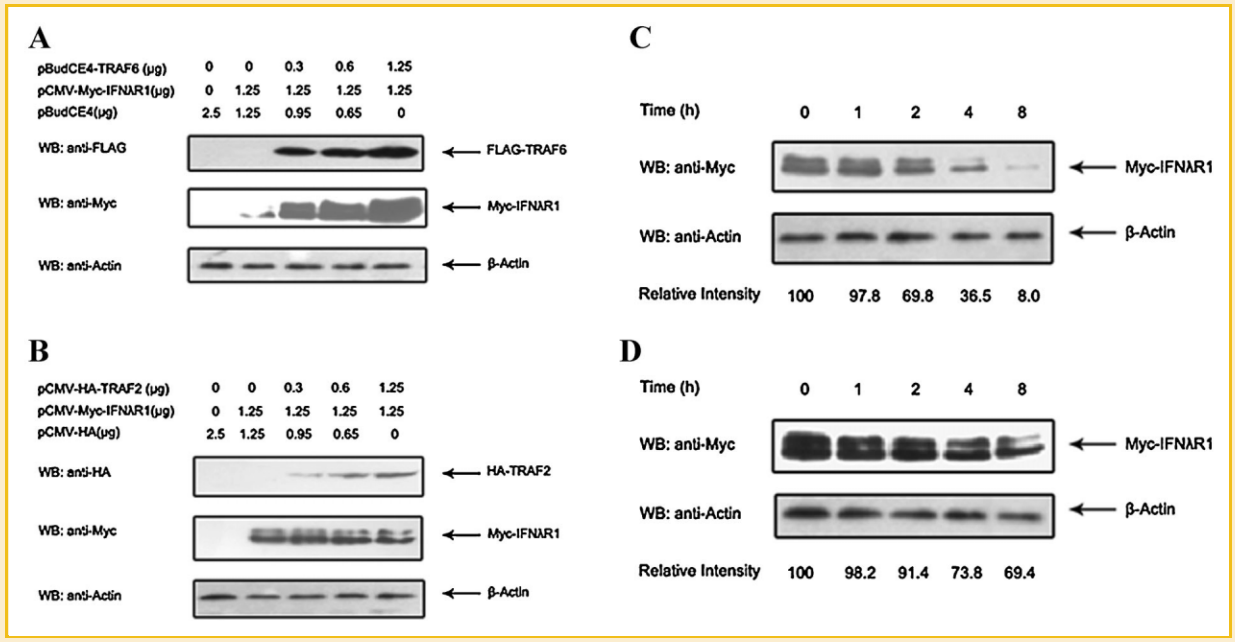

Fig. 5. TRAF6 increases the stability of IFNλR1. A: TRAF6 increases the level of IFNλR1 protein. HEK293T cells were co-transfected with equal amounts of pCMV-Myc-IFNλR1 (1.25 μg) and with or without increasing amounts of pBudCE4-TRAF6 (0.3, 0.6, and 1.25 μg), and the total amount of transfected DNA was adjusted to be constant. Forty-eight hours after transfection, cell lysates were subjected to immunoblotting with the indicated antibodies, while β-actin served as a loading control. B: TRAF2 does not affect the level of IFNλR1 protein. HEK293T cells were co-transfected with the indicated combinations of expression plasmids, and immunoblotting was performed as described above. C, D: TRAF6 increases the half-life of IFNλR1. HEK293T cells were transfected with 1.25 μg pCMV-Myc-IFNλR1 only (C). or co-transfected with 1.25 μg pCMV-Myc-IFNλR1 and 1.25 μg pBudCE4-TRAF6 (D). Forty-eight hours post-transfection, cells were treated with 100 mg/ml cycloheximide for 1, 2, 4, or 8 h. Cells lysates were assayed to detect IFNλR1 with an antibody against c-Myc, and β-actin was used as an internal control. The relative intensity of the bands was quantified using densitometry.

such as CD40 and TRANCE-R (also known as RANK), interact with TRAF6 through this binding site [Ye et al., 2002]. To confirm the association between IFNλR1 and TRAF6, we performed in vitro and in vivo protein–protein interaction assays. Co-immunoprecipitation and GST pull-down assays indicated that the IFNλR1-ICD specifically interacted with TRAF6. Furthermore, Myc-IFNλR1-ICD expressed in HEK293T cells presented two bands that were much larger than the 33 kDa band predicted from its amino acid composition, suggesting that the IFNλR1 ICD was post-translationally modified and that only the 43 kDa IFNλR1 ICD could interact with TRAF6. Post-translational modifications, such as glycosylation, are known to influence protein interaction [Moura et al., 2004]. It is likely that the 45 kDa Myc-IFNλR1-ICD is over-glycosylated, because it can also be stained by the Periodic Acid–Schiff (PAS) reagent (data not shown), leading to a conformational change in IFNλR1 and the abrogation of protein–protein interactions. We further validated the TRAF6-binding site in IFNλR1 and found that residues P480 and E482 are required for the association between IFNλR1 and TRAF6. Next, we explored whether there is an endogenous interaction between TRAF6 and IFNλR1 in some cell lines. Semi-endogenous co-immunoprecipitation experiments revealed that over-expressed IFNλR1 could interact with endogenous TRAF6. In endogenous co-immunoprecipitation assays, IFNλR1 associated with TRAF6, and in the presence of IFNλ1 this association was markedly reduced. The result was similar with the previous report, in which a constitutive interaction between endogenous TβRI and TRAF6 was observed and this was reduced when cells were treated with TGF-β1 for 15 min[Sorrentino et al., 2008]. These data suggest that IFNλ1 mediates the interaction between IFNλR1 and TRAF6, but the underlying mechanism remains unclear.

Because it has the most divergent receptor-binding region in the TRAF family, TRAF6 is unique in its receptor-binding properties. Whereas TRAF1-TRAF5 modulate the signaling of members of the TNF receptor superfamily exclusively, TRAF6 also transduces signals from receptors of the IL-1R/TLR family, NGF and TGF-β [Cao et al., 1996; Khursigara et al., 1999; Sorrentino et al., 2008]. Signal amplification by TRAF6 involves the activation of multiple kinase pathways, including Jak-STAT and NF-κB pathways [Raveh et al., 1996; Kotenko and Pestka, 2000]. NF-κB plays important roles in a variety of physiological and pathophysiological processes. It has been shown to regulate the expression of numerous genes that play critical roles in cell growth, cell survival and apoptosis [Karin and Ben-Neriah, 2000; Karin and Lin, 2002]. Type I IFNs activate NF-κB, promoting cell survival through canonical and non-canonical pathways [Yang et al., 2000, 2005]. It has recently been found that TRAF2 is directly coupled to the signal-transducing IFNAR1 subunit of the type I IFN receptor and plays a critical role in the activation of NF-κB by IFN [Yang et al., 2008]. In comparison with IFNs, TRAF6 is a direct activator of NF-κB signaling. Overexpression of TRAF6 activates NF-κB, and TRAF6 lacking its zinc-binding domains acts as a dominant-negative inhibitor of NF-κB activation [Cao et al., 1996; Ishida et al., 1996]. Our study is the first report of a CRF2 family member, IFNλ that interacts directly

with TRAF6, representing a notable difference from other receptors for IL-10 related cytokines and IFNs.

To determine the function of TRAF6 binding to IFNλR, we conducted luciferase reporter assays. These experiments showed that overexpression of TRAF6 resulted in a marked stimulation of NF-κB reporter activity, which is consistent with previous reports [Xiong et al., 2004; Wang et al., 2011]. However, TRAF6 induced-NF-κB activation was largely inhibited, in a dose-dependent manner, by expression of IFNλR1 in the presence of IFNλ1. We further found that the inhibition was reduced when the TRAF6-binding motif was disrupted. It has been reported that TRAF6 auto-ubiquitination is the critical upstream mediator of NF-κB activation, which may have general implications in other TRAF6-mediated signaling processes [Lamothe et al., 2007]. We hypothesized that IFNλR1 inhibition of NF-κB might result from blockade of TRAF6 ubiquitination. To test this possibility, we conducted in vivo ubiquitination assays. We found that IFNλR1 reduced autoubiquitination of TRAF6. Thus, in summary, our studies reveal that the interaction of IFNλR1 with TRAF6 inhibits TRAF6-induced NF-κB activation, and this inhibition may be due to reduced TRAF6 autoubiquitination.

Moreover, we observed that TRAF6 dramatically enhanced the level of the IFNλR1 ICD protein, which led us to test the possibility that IFNλR1 is stabilized by TRAF6. Confirming this result, the half-life of IFNλR1 protein was markedly increased in the presence of TRAF6. TRAF6 has a similar effect on neurotrophin receptor interacting factor (NRIF) and MAST205 [Gentry et al., 2004; Xiong et al., 2004]. TRAF6 has been found to act as a ubiquitin E3 ligase, which play a pivotal role in ubiquitination. The canonical role of ubiquitination is to mediate degradation. However, protein modification by Ub has much broader and diverse functions involved in a myriad of cellular processes. Ub may function as an internalization signal that sends the modified substrate to the endocytic/sorting compartments, followed by recycling to the plasma membrane or degradation in the lysosome [d'Azzo et al., 2005]. Whether ubiquitination is involved in this process and how IFNλR1 degradation is suppressed by TRAF6 remains to be elucidated.

In conclusion, our studies reveal that the interaction of IFNλR1 with TRAF6 inhibits TRAF6-induced NF-κB activation and increased the half-life of IFNλR1. Our future study is focus on whether IFNλ and its receptor protein is involved in antiviral, antiproliferative, antitumor, and immune responses by interfering with TRAF6-NF-κB signaling pathways.

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