The Role of TRAF2 Binding to the Type I Interferon Receptor in Alternative NFκB Activation and Antiviral Response*

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Type I interferons (IFNs) play critical roles in the host defense by modulating gene expression through the IFN-dependent activation of STAT and NFκB transcription factors. Previous studies established that IFN activates NFκB through a classical NFκB pathway that results in IkB degradation and formation of p50-containing NFκB complexes, as well as an alternative pathway that involves NFκB-inducing kinase and TRAF2, which results in the formation of p52-containing NFκB complexes. In this study, we examined the interaction of TRAF proteins with the type I IFN receptor. We found that TRAF2 was directly coupled to the signal-transducing IFNAR1 subunit of the IFN receptor. By immunoprecipitation, overexpression of epitope-tagged IFNAR1 constructs, and glutathione S-transferase pull-down experiments, we demonstrate that TRAF2 rapidly binds to the IFNAR1 subunit of the IFN receptor upon IFN binding. The membrane proximal half of the IFNAR1 subunit was found to directly bind TRAF2. Moreover, analysis of mouse embryonic fibroblasts derived from TRAF2 knock-out mice demonstrated that TRAF2 plays a critical role in the activation of the alternative NFκB pathway by IFN, but not the classical NFκB pathway, as well as in the antiviral action of IFN. Our results place TRAF2 directly in the signaling pathway transduced through the IFNAR1 subunit of the IFN receptor. These findings provide an important insight into the molecular mechanisms by which IFN generates signals to induce its biological effects.

The family of tumor necrosis factor (TNF) receptor-associated factors (TRAFs) functions as adaptor molecules for TNF superfamily members by associating with the intracellular domain of these receptors to mediate downstream signaling events such as NFκB and AP-1 activation (1). TRAF proteins, originally discovered because of their ability to bind to the p75 TNFR receptor, represent a family of six proteins containing a conserved C-terminal domain, which enables their interaction with various members of the TNF receptor superfamily. Moreover, TRAF proteins can also integrate NFκB activation to signal transduction by other receptors, including the family of Toll-like receptors, the LMP1 protein of Epstein-Barr virus, and lymphotoxin-β (2). TRAF proteins play critical roles in innate immune responses, inflammatory processes, and programmed cell death, which are also processes regulated by the type I IFN family of proteins (IFNα, IFNβ, and IFNω). However, the role of TRAF proteins in the cellular response to IFNs has been explored only recently (3, 4). Type I IFNs bind to a ubiquitously expressed cell-surface receptor comprising the IFNAR1 and IFNAR2 chains. The IFN signal transduction pathway involves the recruitment of STAT proteins to the IFN receptor subunits and STAT activation by receptor-associated Janus kinase tyrosine kinases (5–7).

Besides the classical JAK/STAT signaling pathway, type I IFNs also activate the NFκB transcription factor that regulates the expression of genes involved in cell survival and in immune and inflammatory responses (8–11). In mammals, the NFκB protein family includes NFκB1 (p105 processed to p50), NFκB2 (p100 processed to p52), RelA, RelB, and c-Rel. Although p50/RelA and p52/RelB heterodimers are the NFκB complexes most often observed in cells, other combinations of Rel homodimers and heterodimers also form. The NFκB1 and NFκB2 precursor proteins undergo proteolytic processing into the p50 and p52 proteins, respectively. Although the processing of p105 is constitutive and largely cotranslational, the proteolytic processing of p100 protein results from its ligand-inducible phosphorylation and subsequent ubiquitylation.

In common with a variety of stimuli, IFNα/β promotes the dissociation of the cytosolic inactive NFκB-IκB complexes via the serine phosphorylation and degradation of IκB, leading to NFκB translocation to the nucleus and DNA binding (12), which is denoted as the classical NFκB activation pathway. IFNα/β also induces NFκB activation by an alternative pathway dependent on NFκB-inducing kinase and TRAF proteins that results in the processing of the p100/NFκB2 precursor into p52. We showed previously that expression of a dominant-negative TRAF2 construct inhibited IFN-promoted NFκB activation (8), suggesting a role for TRAF2 in coupling of the IFN receptor signaling to NFκB activation.

In this study, we examined the interaction of TRAF proteins with the type I IFN receptor. We found that TRAF2 binds to the signal-transducing IFNAR1 subunit of the type I IFN receptor.
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By immunoprecipitation, overexpression of epitope-tagged IFNAR1 constructs, and GST pulldown experiments, we demonstrate that TRAF2 rapidly bound to the IFNAR1 subunit upon IFN addition to cells. The interaction of TRAF2 with the IFNAR1 subunit was localized to the membrane proximal portion of the receptor. Furthermore, analysis of mouse embryo fibroblasts (MEFs) derived from TRAF2 knock-out (KO) MEFs demonstrated that TRAF2 plays an important role in coupling alternative NFκB activation to the type I IFN receptor and in the induction of antiviral activity by IFN.

EXPERIMENTAL PROCEDURES

Biological Reagents and Cell Culture—Recombinant human IFNα (IFNaCon1) and rat IFNβ were provided by InterMune (Brisbane, CA) and Biogen Idec, Inc. (Cambridge, MA), respectively. The biological activity of the IFN preparations was expressed in terms of international reference units/ml using the appropriate National Institutes of Health reference standard as described previously (13). Human Daudi cells were cultured in RPMI 1640 medium with 10% bovine calf serum. HT1080 fibroblasts (MEFs) derived from TRAF2 knock-out (KO) MEFs were cultured in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum (14). Wild-type (WT) and TRAF2-KO MEFs were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (15). Cells were maintained in the presence of penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO2.

Constructs and Transfection Conditions—The cDNAs for TRAF2 and IFNAR1 were amplified by PCR and cloned into the pcEF expression vector that provides a c-Myc epitope tag at the C terminus of the protein (7). Transfection of cells (107) was accomplished by electroporation (capacitance of 300 microfarads, 250 V) with 20 μg of plasmid DNA and 500 μg of salmon sperm DNA. Stable transfectants were selected for neomycin resistance (0.4 mg/ml G418).

IkBα Degradation—At various times after IFN (1,000 units/ml) addition, cells pretreated with cycloheximide (30 μg/ml, 1 h) were washed with ice-cold phosphate-buffered saline and lysed in modified radioimmune precipitation assay buffer (phosphate-buffered saline, pH 7.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mm NaF, 1 mm Na3VO4, 1 mm phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1.75 μg/ml benzamidine). Precleared cell lysates (12,000 g, 15 min) were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes, immunoblotted with specific affinity-purified rabbit anti-IkBα antibody (Santa Cruz Biotechnology), and visualized by enhanced chemiluminescence (Pierce).

NFκB DNA-binding Activity Measurements—Nuclei were extracted with buffer (20 mM Tris-HCl, pH 7.85, 250 mM sucrose, 0.4 mM KCl, 1.1 mM MgCl2, 5 mM β-mercaptoethanol, 1 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1.75 μg/ml benzamidine), and extracts were frozen and stored at −80 °C (7). For electrophoretic mobility shift assays (EMSA), the nuclear extracts were incubated with a 32P-labeled κB probe (5′-AGTTGAGGGCAGTCTCCAGG-3′) derived from an NFκB binding sequence in the immunoglobulin gene promoter (16). To define the presence of specific Rel proteins, nuclear extracts were preincubated with a 1:50 dilution of anti-Rel antibodies at 25 °C for 0.5 h and then subjected to EMSA. Gels were quantitated by phosphorimage autoradiography.

Immunoprecipitation and Immunoblot Analysis—For immunoprecipitation studies, cells (1 × 108) were treated with IFN (1,000 units/ml) at 37 °C for the indicated periods of time, washed with ice-cold phosphate-buffered saline, and incubated for 20 min in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 15% glycerol) containing 1 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1.75 μg/ml benzamidine. Samples were centrifuged (12,000 × g, 15 min) at 4 °C, and supernatants (equivalent to 1 × 107 cells) were immunoprecipitated with the indicated antibody overnight at 4 °C. Immune complexes were collected using protein A-Sepharose beads (GE Healthcare), washed extensively, and eluted in Laemmli buffer. Samples were subjected to SDS-PAGE, transferred to PVDF membranes (Millipore), and probed with the indicated antibody followed by anti-mouse IgG coupled with horseradish peroxidase (Santa Cruz Biotechnology) or mouse IgG TrueBlot (eBioscience). Blots were developed using enhanced chemiluminescence (Pierce).

GST Fusion Constructs and GST Pulldown Assays—cDNAs encoding amino acids 462–557 (AR1), 462–507 (AR1N), and 507–557 (AR1C) of the IFNAR1 intracellular domain were amplified from IFNAR1 cDNA and cloned into the EcoRI and XhoI sites of pGEX-KG, which provides the proteins with an amino-terminal GST tag (17). GST fusion proteins were expressed in Escherichia coli strain BL21(DE3) by transformation with the plasmid construct and affinity-purified on glutathione-Sepharose (GE Healthcare) as described previously (18). For GST pulldown assays, lysates from control or IFN-treated (~2 × 107) cells were incubated with GST fusion proteins bound to glutathione-agarose beads at 4 °C overnight. The bound proteins were washed extensively and eluted with Laemmli buffer, resolved by SDS-PAGE (7.5%), blotted onto PVDF membranes, and probed as indicated.

Antiviral and Antiproliferative Assays—To determine antiviral activity, MEF cultures were incubated overnight with rat IFNβ, followed by infection with vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) for 2 h at 0.1 plaque-forming units/cell. At 24 h post-infection, the VSV yield in the medium was assayed by plaque formation on Vero cells as described previously (11). At 24 h post-infection, the cytopathic effect of EMCV on STAT1-KO MEF cultures was determined by uptake of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as a measure of cell viability. To determine antiproliferative activity, MEF cultures were plated at 1 × 105 cells in 25-cm2 flasks and treated with rat IFNβ. 3 days after IFN addition, cells were harvested by trypsinization and enumerated in a Coulter Counter (19).

RESULTS

Binding of TRAF2 to the IFNAR1 Chain of the Type I IFN Receptor—We reported previously that expression of a dominant-negative TRAF2 construct in the highly IFN-sensitive human Daudi lymphoblastoid cell line blocked IFN-promoted
NFκB DNA-binding activity and p100 processing (8). Because TRAF proteins by definition are receptor-associated factors, we investigated whether TRAF2 is associated with the IFN receptor. Lysates were prepared from control and IFN-treated (1,000 units/ml, 15 min) Daudi cells, immunoprecipitated with antibodies directed against the extracellular domains of the IFNAR1 or IFNAR2 subunit (20), and then immunoblotted with Abs directed against TRAF2. As shown in Fig. 1A, although TRAF2 was found not to be basally associated with either the IFNAR1 or IFNAR2 chain of the IFN receptor, IFN treatment induced the association of TRAF2 with the IFNAR1 subunit, but not with the IFNAR2 subunit. Next we examined the time course of TRAF2 association with IFNAR1. Daudi cells were treated with IFN for varying times, lysed, and immunoprecipitated with anti-IFNAR1 antibody. As shown in Fig. 1B, IFN induced the association of TRAF2 with the IFNAR1 subunit within 5 min, and TRAF2 remained bound to IFNAR1 up to 1 h after IFN addition. In contrast, although TRAF6 was expressed in Daudi cells, TRAF6 was not bound to IFNAR1 either before or after IFN treatment (data not shown). The results demonstrate that IFN induces the specific association of TRAF2 with the IFNAR1 subunit of the IFN receptor. IFN signaling involves both JAK-mediated tyrosine phosphorylation and PI3K/Akt-mediated serine phosphorylation. To determine whether phosphorylation plays a role in the IFN-induced interaction of IFNAR1 with TRAF2, Daudi cells were treated with genistein (a serum kinase inhibitor) or LY294002 (a PI3K inhibitor) as we have used previously (10, 13, 21). As shown in Fig. 1C, inhibitors of either IFN-activated signaling pathway reduced the IFN-induced interaction of IFNAR1 with TRAF2. However, this approach does not discern whether phosphorylation of TRAF2 and/or IFNAR1 is involved in this interaction, which is addressed by GST pulldown assays.

Role of the Intracellular Domain of IFNAR1 in IFN-dependent TRAF2 Binding—TRAF proteins mediate signal transduction by interacting with the intracellular domain of various members of the TNF receptor superfamily. To further characterize the interaction of TRAF2 with the IFNAR1 subunit, Daudi cells were transiently transfected with various Myc epitope-tagged IFNAR1 constructs. 48 h after transfection, cell lysates prepared from control and IFN-treated (1,000 units/ml, 15 min) Daudi cells were immunoprecipitated with anti-c-Myc antibodies and immunoblotted for IFNAR1 or TRAF2. As shown in Fig. 2A, IFN induced the association of TRAF2 with full-length IFNAR1, but not with IFNAR1 without its intracellular domain. Full-length and intracellular domain-deleted IFNAR1 were expressed at similar levels in transiently transfected Daudi cells. These results demonstrate that the interaction of TRAF2 with IFNAR1 is IFN-dependent and localize the site of TRAF2 interaction to the intracellular domain of IFNAR1.

To define the region of the intracellular domain of IFNAR1 required for TRAF2 binding, various portions of the IFNAR1 chain were expressed as GST fusion proteins, and GST pulldown assays were performed. Daudi and HT1080 fibrosarcoma cells were treated with IFN (1,000 units/ml, 15 min), cell extracts were prepared and incubated with GST fusion proteins, and GST-bound material was immunoblotted for TRAF2 or GST. As shown in Fig. 2B, none of the IFNAR1-GST constructs bound TRAF2 in extracts prepared from control cells. However, the full-length IFNAR1 intracellular domain as well as the membrane proximal, amino-terminal half of the intracellular domain of IFNAR1 (AR1m) bound TRAF2 from extracts prepared from IFN-treated Daudi cells or HT1080 cells (Fig. 2B). TRAF2 was not bound to the carboxyl-terminal half of the IFNAR1 intracellular domain (AR1c) in extracts prepared from control or IFN-treated cells, although the various IFNAR1-GST constructs were present at equivalent amounts. These results demonstrate that the membrane proximal half of the intracellular domain of the IFNAR1 chain directly binds TRAF2 upon IFN treatment. Pretreatment of Daudi cells with genistein or LY294002 blocked the IFN-induced binding of TRAF2 to the full-length IFNAR1 intracellular domain-GST construct (Fig. 2C). Moreover, because the GST constructs do not undergo IFN-induced phosphorylation, the results presented in Figs. 1 and 2 indicate that upon IFN treatment, phosphorylated TRAF2 binds to the membrane proximal half of IFNAR1.

Activation of the Alternative NFκB Pathway Is Directed through the IFNAR1 Chain—IFN activates a TRAF-dependent alternative NFκB pathway, which results in the formation of p52-p65 NFκB complexes (8). We established previously that when expressed in mouse L cells, the human IFNAR1 chain functions as a species-specific transducer for human IFN (14, 22). Utilizing this system, we examined whether signaling mediated through the intracellular domain of IFNAR1 is required for IFN-induced p100 processing. Mouse L cells expressing either full-length human IFNAR1 or an IFNAR1 chain lacking the intracellular domain were treated with human IFNα (1,000 units/ml, 15 min), assayed by pulldown assays with p65-GST, and immunoblotted with p52. As shown in Fig. 3A, IFN induced the formation of p52-p65 complexes in mouse cells expressing full-length human IFNAR1, but not in cells transfected with the human IFNAR1 chain lacking the intracellular domain or an empty vector construct. Moreover, p52-p65 complexes were not detected in either transfectant...
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![Diagram](image)

**FIGURE 2. Binding of TRAF2 to the intracellular domain of the IFNAR1 chain.** A, Daudi cells were transiently transfected (transfection efficiency of ~85%) for 48 h with the Myc epitope-tagged full-length IFNAR1 subunit (AR1), an IFNAR1 subunit with the intracellular domain deleted (AR1ΔIC), or empty vector (EV). Lysates were prepared from IFN-treated cells (1,000 units/ml, 15 min) and immunoprecipitated with anti-Myc epitope Abs. The precipitates were resolved by SDS-PAGE, blotted onto PVDF membranes, probed with anti-TRAF2 or anti-IFNAR1 antibody as indicated, and visualized by enhanced chemiluminescence. B, cell lysates were prepared from WT or TRAF2-KO MEFs treated with rat IFNβ (1,000 units/ml, 15 min) and after IFN treatment for 30 min, cell lysates were incubated with anti-TRAF2 or anti-GST antibody. Blots were visualized by enhanced chemiluminescence. C, Daudi cells were treated with IFN (1,000 units/ml) or TNF (10 ng/ml) for varying times and subjected to EMSA. D, nuclear extracts were prepared from Daudi cells treated with IFN (1,000 units/ml, 30 min) and incubated with anti-p50 or anti-p65 antibody or a 50-fold excess of unlabeled κB oligonucleotide probe (cold) prior to EMSA.

in the absence of IFN treatment. These results demonstrate 1) the species specificity of the interaction between the human IFNAR1 chain and human IFN and 2) that the intracellular domain of the human IFNAR1 is required for induction of the alternative NFκB signaling pathway by human IFN.

**Role of TRAF2 in IFN-induced NFκB Signaling Pathways—** To further define the role of TRAF2 in the IFN-induced activation of the NFκB signaling pathway, we employed fibroblasts derived from TRAF2-KO MEFs. IFN induces IkBα degradation (the classical NFκB pathway), as well as an alternative NFκB pathway that is independent of IkBα degradation (10). To examine the contribution of TRAF2 to the classical NFκB pathway, IFN-promoted IkBα degradation was assessed in extracts prepared from WT and TRAF2-KO MEFs treated with IFN for varying times. As shown in Fig. 3B, IFN induced IkBα degradation with a similar time course in both WT and TRAF2-KO MEFs, indicating that TRAF2 is not required for IFN-induced IkBα degradation. To determine whether TRAF2 is required for induction of the NFκB-dependent DNA binding, TRAF2-KO MEFs were stimulated with IFNβ, and NFκB activation was examined by EMSA with a consensus κB oligonucleotide probe. As shown in Fig. 3C, IFN induced NFκB DNA-binding activity within 30 min, and activity returned to near baseline levels by 2 h after IFN addition. The kinetics of NFκB activation are similar to what we have observed previously in IFN-treated MEFs (11). Because TNF also induces NFκB activity via the classical pathway (23), TRAF2-KO MEFs were treated with TNF for comparison. As shown in Fig. 3C, TNF also induced NFκB activation in TRAF2-KO MEFs. However, the kinetics of NFκB induction by TNF, as well as the NFκB complexes
formed, were clearly different from those induced by IFN treatment. In addition, supershift assays with Rel antisera demonstrate the induction of p50-containing NFκB complexes in nuclear extracts of IFN-treated TRAF2-KO MEFs (Fig. 3D). Moreover, anti-p52 Ab caused no supershift of IFN-induced complexes in TRAF2-KO cells (data not shown). Taken together, these results demonstrate that TRAF2 is not required for the activation of the classical NFκB signaling pathway by IFN.

To directly examine the contribution of TRAF2 to the alternative NFκB pathway, IFN-promoted formation of p52-p65 complexes was assessed by p65-GST pulldown assays with extracts prepared from WT and TRAF2-KO MEFs treated with IFN for varying times. As shown in Fig. 4A, whereas IFN induced the formation of p52-p65 complexes in WT MEFs, these NFκB complexes were not detected in IFN-treated TRAF2-KO MEFs. Immunoblotting with p50 did not detect the formation of p50-p65 complexes. To confirm the role of TRAF2 in this pathway, TRAF2-KO MEFs were reconstituted with TRAF2. As shown in Fig. 4A, the level of TRAF2 expression in TRAF2-reconstituted TRAF2-KO MEFs was similar to that observed in WT MEFs. Most importantly, reconstitution of TRAF2-KO cells with TRAF2 restored the IFN-induced formation of p52-p65 complexes. These results demonstrate that formation of p52-p65 complexes induced by IFN is TRAF2-dependent.

The JAK/STAT pathway is critical in IFN signaling. To determine whether TRAF2 affects JAK/STAT signaling, we also assessed STAT activation by IFN in WT and TRAF2-KO MEFs. As shown in Fig. 4B, IFN-induced STAT1, STAT2, and STAT3 activation as determined by tyrosine phosphorylation was indistinguishable between WT and TRAF2-KO MEFs. Moreover, the kinetics of IFN-induced STAT1 activation were similar in WT and TRAF2-KO MEFs (Fig. 4C). Similar kinetics of IFN-induced STAT2 and STAT3 activation were also observed in WT and TRAF2-KO MEFs (data not shown). Taken together, these results illustrate the selective role that TRAF2 plays in IFN signaling: TRAF2 is required for the induction of the alternative NFκB pathway by IFN but is not required for either the IFN-induced JAK/STAT pathway or the classical NFκB pathway.

Role of TRAF2 in Biological Effects Induced by IFN—We next assessed the role of TRAF2 in the induction of the biological actions of IFN. To determine the role of TRAF2 in the antiproliferative activity of IFN, WT and TRAF2-KO MEFs were cultured for 3 days in the presence of varying concentrations of IFN. As shown in Fig. 5A, the proliferation of WT and TRAF2-KO MEFs was inhibited by IFN treatment to a similar extent, with an ~30% inhibition observed at 100 units/ml IFN. We then assessed the sensitivity of WT and TRAF2-KO MEFs to the antiviral action of IFN by determining the ability of IFN to reduce VSV replication. As shown in Fig. 5B, whereas 10 units/ml IFN markedly protected WT MEFs from virus-induced cell death, it was not effective in TRAF2-KO MEFs. VSV induced cell death to a similar extent in untreated WT and TRAF2-KO MEFs. Moreover, the ability of IFN to inhibit virus replication was markedly impaired in TRAF2-KO MEFs at the IFN concentrations examined (Fig. 5C). To substantiate whether TRAF2 plays an important role in the ability of IFN to inhibit virus replication, we performed additional experiments in which the antiviral action of IFN against EMCV was assayed.
in WT and TRAF2-KO MEFs. MEFs were treated with various IFN concentrations and infected with EMCV, and virus produced was assayed for the ability to induce cytopathicity in MEFs derived from STAT1-KO mice. STAT1-KO MEFs were employed to eliminate the possible induction of the IFN system during EMCV infection, as they are highly resistant to the antiviral action of IFN (24). As shown in Fig. 5D, IFN inhibited the replication of EMCV to a greater extent in WT MEFs than in TRAF2-KO MEFs. For example, treatment with only 3 units/ml IFN reduced EMCV replication in WT-MEFs to an extent equivalent to IFN treatment with 30 units/ml in TRAF2-KO MEFs, representing a 10-fold difference in IFN sensitivity. It is of interest that the resistance of TRAF2-KO MEFs to the antiviral action of IFN was overcome at high IFN concentrations. These results indicate that TRAF2 plays an important role in the antiviral activity of IFN but is dispensable in the antiproliferative action of IFN.

DISCUSSION

TRAF2 is an important signal transducer for a wide range of TNF receptor superfamily members (TNFR1, TNFR2, p75<sup>NTR</sup>, CD40, etc.), as well as lymphocyte costimulatory receptors (LMP1, CD27, 4-1BB, etc.) (25). Type I IFNs bind to a ubiquitously expressed receptor that comprises two subunits,
IFNAR1 and IFNAR2. Previous studies have demonstrated that IFNAR1 does not directly bind IFN but functions as a species-specific signal transducer for type I IFNs (22). Although the role of IFNAR1 in the JAK/STAT pathway in IFN signal transduction has been characterized (7, 26, 27), its role in integrating IFN-induced NF-κB activation has not been defined. In this study, we have shown that IFN induces the binding of TRAF2 to the intracellular domain of the IFNAR1 chain by coimmunoprecipitation, transfection of epitope-tagged IFNAR1 constructs, and GST pulldown assays. Our finding that TRAF proteins were not associated with the IFNAR2 ligand-binding subunit of the IFN receptor evidenced the specificity of the interaction of TRAF2 with IFNAR1.

TRAF2 binds to the intracellular domains of TNF receptor superfamily members as well as other proteins to mediate NF-κB activation. We have demonstrated that IFN induces TRAF2 binding to the amino-terminal half of the intracellular domain of IFNAR1. Interestingly, this is the first interaction of a signaling protein in the IFN system to dock to this portion of IFNAR1. We found previously that the carboxyl-terminal half of IFNAR1 was involved in STAT3 binding to the IFNAR1 chain (7). IFN activation of the classical NF-κB pathway involves STAT3, which acts as an adaptor for the PI3K/Akt pathway that results in classical NF-κB activation (10). It is tempting to speculate that the IFNAR1 chain directs both classical and alternative NF-κB pathways. The alternative pathway is directed through the amino-terminal half, whereas the carboxyl-terminal half directs the classical pathway. Although IFNAR1 knock-out mice have been derived, the role of IFNAR1 in NF-κB activation has not yet been examined. An SXSE motif has been identified as a TRAF2-binding consensus sequence in several TNF receptor superfamily members (28). A single conserved SXSE motif (SIDE at amino acids 477–480) is present in the amino-terminal intracellular domain of human IFNAR1 that is present in multiple IFNAR1 homologs (mouse, bovine, ovine, and human chains). In studies with the TNF receptor superfamily, TRAF2 association with receptors is constitutive, but we found that TRAF2 association with IFNAR1 association is dependent on IFN treatment, which is blocked by tyrosine kinase and PI3K inhibitors. Our results are consistent with the hypothesis that IFN-induced serine/threonine phosphorylation of TRAF2 through the PI3K/Akt pathway is required for TRAF2 binding to the IFNAR1 subunit of the IFN receptor. IFN induces the tyrosine and serine phosphorylation of IFNAR1 as well as other signaling intermediates, i.e. STAT proteins, that affect IFN action (7, 22, 26, 29). Moreover, the phosphorylation of TRAF2 at Thr117 is induced by TNF and is required for TRAF2 function (30). However, preliminary studies using a TRAF2(T117A) mutant do not implicate a role for the phosphorylation of this TRAF2 residue in IFN signaling. Therefore, it will be important in future studies to elucidate the role of IFN-induced phosphorylation of TRAF2 in binding to the IFNAR1 subunit of the IFN receptor.

Many cytokines promote the dissociation of the cytosolic inactive NF-κB-IκB complexes via the serine phosphorylation and degradation of IκB, leading to NF-κB translocation to the nucleus and DNA binding (12), which is denoted as the classical NF-κB pathway. The degradation of IκB proteins requires the activation of the IκB kinase (IKK), a multiprotein complex consisting of IKKα and IKKβ catalytic subunits and the IKKγ/NF-κB essential modulator regulatory subunit (31). Targeted gene disruption of individual IKK proteins has determined that IKKβ and IKKγ (but not IKKα) are the major mediators of the classical NF-κB pathway (32–36). TRAF2-mediated ubiquitylation of the TAK1 kinase, an upstream regulator of IKKs, is important in activating the classical NF-κB pathway (37). Recent studies have identified that the LMP1 protein of Epstein-Barr virus, B cell-activating factor, lymphotoxin-β, and lipopolysaccharide induce NF-κB activation through an NF-κB signaling pathway that does not involve IκB degradation (38–46). This alternative pathway of NF-κB activation involves the linkage of TRAF2 to the activation of the MAP3K-related kinase, NF-κB-inducing kinase, which results in the ubiquitylation and proteolytic processing of the p100/NFκB2 protein and nuclear translocation of p52/Rel dimers to regulate NFκB target genes (47). Although TRAF2 plays a critical role in the alternative NFκB pathway, both positive and negative regulatory roles of TRAF2 have been described (48, 49).

We have shown by several independent means (TRAF2-KO MEFs and expression of human IFNAR1 in heterologous mouse L cells) that TRAF2 is required for IFN-induced activation of the alternative NFκB pathway, but not of the classical pathway. These results are consistent with our previous finding that a dominant-negative TRAF2 construct blocked the formation of p52/p65 dimers but had no effect on IFN-induced IκB degradation (8). In contrast to the requirement of TRAF2 for the activation of the alternative NFκB pathway, we found that TRAF2 does not affect the activation of the JAK/STAT signaling pathway by IFN, as IFN activates STAT1, STAT2, and STAT3 in TRAF2-KO MEFs.

These results also demonstrate the distinct role that TRAF2 plays in the biological activities of IFN. Although the ability of IFN to inhibit the proliferation of WT and TRAF2-KO MEFs was similar, the induction of the antiviral activity of IFN against two different viruses, VSV and EMCV, was markedly reduced in TRAF2-KO MEFs. Moreover, in preliminary studies, we found that TRAF2 also plays a complex role in regulating IFN-induced gene expression, with some genes induced at higher levels in TRAF2-KO MEFs and others unaffected or induced at lower levels (data not shown). We showed previously that NFκB plays an important and complex role in regulating IFN-induced gene expression (11, 50). Because IFN-induced STAT activation is unaffected in TRAF2-KO MEFs, the differences in gene expression observed probably reflect the binding of different NFκB complexes to gene promoters. However, it is presently unknown which genes are regulated by p52-containing NFκB complexes.

In conclusion, our studies add a new facet to our understanding of how IFNs generate signals. TRAF2 is bound to the amino-terminal domain of the IFNAR1 chain upon IFN addition. TRAF2 mediates the IFN-induced alternative NFκB pathway. Although TRAF2 is dispensable for STAT activation and induction of antiproliferative activity, TRAF2 is important in the induction of the antiviral activity of IFN.
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