Role of Tyrosine 441 of Interferon-γ Receptor Subunit 1 in SOCS-1-mediated Attenuation of STAT1 Activation*

Suppressor of cytokine signaling (SOCS)-1, the key negative regulator of interferon (IFN)-γ-dependent signaling, is induced in response to IFNγ. SOCS-1 binds to and inhibits the IFNγ receptor-associated kinase Janus-activated kinase Janus-activated kinase (JAK) 2 and inhibits its function in vitro, but the mechanism by which SOCS-1 inhibits IFNγ-dependent signaling in vivo is not clear. Upon stimulation, mouse IFNγ receptor subunit 1 (IFNGR1) is phosphorylated on several cytoplasmic tyrosine residues, and Tyr419 is required for signal transducer and activator of transcription (STAT) 1 activation in mouse embryo fibroblasts. However, the functions of the other three cytoplasmic tyrosine residues are not known. Here we show that Tyr441 is required to attenuate STAT1 activation in response to IFNγ. Several tyrosines to phenylalanine mutants of IFNGR1, expressed at normal levels in stable pools of IFNGR1-null cells, were analyzed for the phosphorylation of STAT1 during a 48-h period, and antiviral activity in response to IFNγ was also measured. Stronger activation of STAT1 was observed in cells expressing all IFNGR1 variants mutated at Tyr441, and, consistently, stronger antiviral activity was also observed in these cells. Furthermore, constitutive overexpression of SOCS-1 inhibited IFNγ-dependent signaling only in cells expressing IFNGR1 variants that included the Tyr441 mutation. Mutation of Tyr441 also blocked the ability of SOCS-1 to bind to IFNGR1 and JAK2 in response to IFNγ and the normal down-regulation of STAT1 activation and antiviral activity. These results, together with data from the literature, suggest a model in which, in response to IFNγ, phosphorylation of Tyr441 creates a docking site for SOCS-1, which then binds to JAK2 within the receptor-JAK complex to partially inhibit JAK2 phosphorylation. Furthermore, the virtually complete blockade of STAT1 phosphorylation by overexpressed SOCS-1 in this experiment suggests that the binding of SOCS-1 to Tyr441 also blocks the access of STAT1 to Tyr419 and that this effect may be the principal mechanism of inhibition of downstream signaling.

Interferon (IFN)γ plays key roles in mediating antiviral and antiproliferative responses (1). The major signal transduction pathway activated by IFNγ has been elucidated through both biochemical and genetic studies. The IFNγ receptor complex consists of two receptor subunits, IFNGR1 and IFNGR2, and the tyrosine kinases Janus-activated kinase (JAK) 1 and JAK2, which bind to IFNGR1 and IFNGR2, respectively. IFNγ induces the oligomerization of the receptor subunits, leading to the activation of JAK1 and JAK2, which then phosphorylate tyrosine residues within the cytoplasmic domain of IFNGR1. Signal transducer and activator of transcription (STAT) 1 is then recruited to the receptor complex and phosphorylated on Tyr701, allowing it to be released, form homodimers, translocate to the nucleus, and bind to γ-activated sequences to activate the transcription of interferon-stimulated genes (ISGs) (1–3).

The activation of STAT1 by IFNγ is tightly controlled by several mechanisms (4). The SH2-containing phosphatase 2 binds to IFNGR1 and inhibits STAT1 activation without inhibiting the phosphorylation of IFNGR1 (5). Protein inhibitor of activated STAT 1 (PIAS-1) binds to STAT1 and prevents its association with target DNA (6). Both genetic and biochemical studies have shown that suppressor of cytokine signaling (SOCS)-1 is the most potent inhibitor of IFNγ signaling (7). Mice lacking SOCS-1 develop a complex fatal neonatal disease (8–10), and the mortality, which results from hypersensitivity to IFNγ, is largely prevented by administration of anti-IFNγ. In addition, premature death does not occur in mice lacking both SOCS-1 and IFNγ (9, 11). In response to IFNγ, STAT1 activation is much stronger in cells lacking SOCS-1 than in wild-type cells (11, 12). The constitutive expression of SOCS-1 blocks IFNγ-mediated antiviral and antiproliferative activities (13), and in vitro studies have shown that the SOCS-1 protein inhibits the kinase activity of JAK2 by binding directly to the active site loop domain (14). SOCS-1 can also direct proteins to which it binds, including guanine nucleotide exchange factor VAV, insulin receptor substrates 1 and 2, and JAK2, to proteasome-mediated degradation (15–18).

Tyrosine phosphorylation is used by most cell surface receptors to initiate downstream signaling in response to cytokines and growth factors (19–23). Receptor phosphorylation creates docking sites for downstream signaling components and mutation of specific tyrosine residues blocks signal transduction (24–28). The situation for IFNγ-dependent signaling has been well studied. Activation of JAK1 and JAK2 is mediated by trans- and auto-phosphorylation. Phosphorylated Tyr419 of IFNGR1 creates the docking site for STAT1 in mouse embryo fibroblasts (MEFs), and phosphorylation of Tyr440 of human IFNGR1 has a major role in activating STAT1 and mediating kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling; MEF, mouse embryo fibroblast; ISG, interferon-stimulated gene; SH, Src homology.

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antiviral activity. Tyrosine to phenylalanine mutation of this motif impairs STAT1 activation as well as the expression of ISGs (29, 30).

Tyrosine phosphorylation also provides the basis for negative regulation of receptor-dependent signaling (31–34). For the interleukin-6 family of cytokines, the gp130 receptor subunit is phosphorylated, and four of its cytoplasmic tyrosine residues are involved in the activation of STATs 1 and 3 (35). Furthermore, Tyr759 is required for the binding of SH2-containing phosphatase 2 and SOCS-3 (36, 37). SH2-containing phosphatase 2 mediates the activation of mitogen-activated protein kinase in pro-B cell lines (38) and negatively regulates STAT activation in gp130-dependent signaling (39); SOCS-3 is the key negative regulator of gp130-dependent signaling (40–42).

Mutation of Tyr749 to phenylalanine enhances signal transduction in response to gp130-linked cytokines, including interleukin-6, leukemia inhibitory factor, and oncostatin M (43, 44). Furthermore, mice expressing a gp130 mutant lacking Tyr759 have splenomegaly, lymphadenopathy, and an enhanced acute phase reaction (45). Here, we investigate the involvement of specific tyrosine residues of IFNGR1 in IFN-γ-dependent signaling and find that Tyr441 is required for attenuation. Tyrosine to phenylalanine mutation of this residue leads to stronger STAT1 activation and antiviral activity, and inhibition of signaling in response to SOCS-1 requires Tyr441, as does the IFN-γ-dependent binding of SOCS-1 to IFNGR1 and JAK2.

**EXPERIMENTAL PROCEDURES**

**Constructs—**Plasmids expressing murine IFNGR1 and the IFNGR1 mutant Y419F were kindly provided by Dr. Robert Schreiber (Washington University, St. Louis, MO). IFNGR1 and IFNGR1 Y419F cDNAs were subcloned into pBABEpuroG. Tyrosine to phenylalanine mutations of IFNGR1 were generated by PCR-splicing overlapping extension (46). The identity of each plasmid was confirmed by DNA sequencing. The plasmid encoding SOCS-1, kindly provided by Dr. Ke Shuai (University of California Los Angeles, Los Angeles, CA), was used to subclone this gene into pBluescript at the XbaI site and then into pLHCG at the HindIII and HpaI sites.

**Biological Reagents and Cell Culture—**Recombinant murine IFN-γ (PeproTech, Inc., Rocky Hill, NJ) was used at 1000 IU/ml. Bosc cells (American Type Culture Collection), wild-type MEFs, and IFNGR1-null MEFs (from Dr. Robert Schreiber) (47) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Virus-infected cells were maintained in complete medium plus 2 μg/ml puromycin or 100 μg/ml hygromycin.

**Western Analyses—**After treatment, cells at 80% confluence in 100-mm dishes were washed once with phosphate-buffered saline, and the cell pellets were lysed for 20 min at 4 °C in 100 μl of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin. Cellular debris was pelleted by centrifugation at 16,000 × g for 10 min. Cell lysates were incubated with anti-FLAG antibody and protein G-Sepharose (Amersham Biosciences) overnight. Immunoprecipitates were washed four times with ice-cold lysis buffer and analyzed by the Western method.

**RESULTS**

**Role of Tyr441 of IFNGR1**

**Tyrosine Residues of IFNGR1 Provide Negative Regulation of IFN-γ-dependent Signaling—**Mouse IFNGR1 has four cytoplasmic tyrosine residues, which are phosphorylated upon stimulation with IFN-γ. Tyr441 is required for the activation of STAT1 (46, 47), but the functions of the other tyrosines are not known. Previous studies have shown that Tyr759 of gp130, which is not needed for STAT3 activation, is required for the negative regulation of this process (36, 37, 39). To reveal whether a tyrosine residue of IFNGR1 other than Tyr419 is similarly required for the negative regulation of IFN-γ-dependent signaling, IFNGR1-null cells expressing either wild-type or 3F/419Y IFNGR1 (three of the four cytoplasmic tyrosine residues were mutated to phenylalanines) were used to examine the phosphorylation on tyrosine of STAT1, which was stronger in cells expressing 3F/419Y than in cells expressing wild-type IFNGR1 (Fig. 1a). Because phosphorylated STAT1 is essential for the transcription of most IFN-γ-induced genes, the expression of three ISGs was also examined. After treatment with IFN-γ, the levels of irf1, irf-1, and socs-3 mRNAs were increased by 2-fold or more in cells expressing 3F/419Y compared with cells expressing wild-type IFNGR1 (Fig. 1b). Therefore, STAT1 activation is negatively regulated by a tyrosine residue other than Tyr419 of IFNGR1.

**Ty441 of IFNGR1 Is Required for Negative Regulation of STAT1 Activation in Response to IFN-γ—**To determine which tyrosine(s) might be required, several different tyrosine to phenylalanine mutants were generated (Fig. 2). In mutant 2F/441Y/419Y, STAT1 phosphorylation was attenuated normally,
different mutants, 8 of which were used in this study. The four cytoplasmic tyrosines of IFNGR1, which are conserved between human and mouse, were mutagenized to produce 16 mutants studied. The four cytoplasmic tyrosines of IFNGR1, which are cytoplasmic tyrosine to phenylalanine mutants of IFNGR1, and pools of cells stably expressing the different IFNGR1s were selected. Total cell lysates, together with lysates from wild-type (WT) MEFs, were analyzed by the Western method using antibodies against IFNGR1 and actin.

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expression of various mutants of IFNGR1, and pools of cells stably expressing the different IFNGR1s were selected. Total cell lysates, together with lysates from wild-type (WT) MEFs, were analyzed by the Western method using antibodies against IFNGR1 and actin.

and the attenuation was abolished in all mutants that include Y441F (Fig. 3a). Note that the basal levels of STAT1 in these cells are very low. Detailed time courses with single tyrosine to phenylalanine mutants confirmed that Tyr$^{441}$ is the primary residue that mediates negative regulation of STAT1 activation in response to IFNγ (Fig. 3b). To test whether Tyr$^{441}$ is also involved in modulating the antiviral response, an assay was performed to assess the ability of IFNγ to protect cells from the cytopathic effects of encephalomyocarditis virus in cells expressing wild-type IFNGR1 or several mutants. Cells expressing the 3F/419Y, 2F/285Y/419Y, or 2F/370Y/419Y mutants of IFNGR1 were protected at much lower doses of IFNγ than were cells expressing wild-type IFNGR1 or the 2F/441Y/419Y mutant (Fig. 3c). These data provide additional evidence that Tyr$^{441}$ of IFNGR1 mediates the negative regulation of IFNγ-dependent signaling, including antiviral activity.

Inhibition of IFNγ-dependent Signaling by SOCS-1 Is Mediated by Tyr$^{441}$—Previous studies identified SOCS-1 as a critical inhibitor of IFNγ-dependent signaling (7); SOCS-1 was expressed constitutively in cells with wild-type IFNGR1 or the 3F/419Y, 2F/441Y/419Y, and 3Y/441F mutants, and responses to IFNγ were examined. SOCS-1 blocked STAT1 phosphorylation virtually completely, but only in cells expressing wild-type IFNGR1 or the 2F/441Y/419Y mutant, and not in cells expressing the 3F/419Y or 3Y/441F mutants (Fig. 4a). Constitutive overexpression of SOCS-1 attenuated the IFNγ-dependent phosphorylation of JAK2 by about 2-fold in cells expressing wild-type IFNGR1 or the 2F/441Y/419Y mutant, but not in cells expressing the 3F/419Y or 3Y/441F mutants, and the phosphorylation of JAK1 was intact (Fig. 4b). Protein levels of JAK1 and JAK2 did not change during the time course investigated (data not shown).

Previous studies have shown that SOCS-1-null mice are more resistant to viral infection than are wild-type mice (11) and, conversely, that overexpression of SOCS-1 completely blocks the antiviral activity of IFNγ (13). Constitutively overexpressed SOCS-1 abrogated antiviral responses in cells expressing wild-type IFNGR1 or the 2F/441Y/419Y mutant but did not inhibit this response in cells carrying the 3F/419Y or 3Y/441F mutants of IFNGR1. Consistent with these results, IFNγ protected cells expressing the 3F/419Y or 3Y/441F mutants of IFNGR1 at lower doses than cells with wild-type IFNGR1 or the 2F/441Y/419Y mutant (Fig. 4c). These results indicate that Tyr$^{441}$ is required to mediate the inhibitory effect of SOCS-1 in IFNγ-dependent signaling.

Tyr$^{441}$ Is Required for the IFNγ-dependent Association of SOCS-1 and IFNGR1—The results cited above show that inhibition of IFNγ-dependent signaling by SOCS-1 requires Tyr$^{441}$ of IFNGR1. Does SOCS-1 bind to IFNGR1, and, if so, does binding require Tyr$^{441}$? Coimmunoprecipitation Western
Role of Tyr<sup>441</sup> of IFNGR1

**Contribution of Specific Tyrosine Residues of IFNGR1 to IFNγ-dependent Signaling—**In response to IFNγ, the phospho-tyrosine residues of IFNGR1 have not been investigated until now. Here we show that an important function of one of these residues is attenuation of IFNγ-induced antiviral responses, which are stronger in IFNGR1-null MEFs expressing mutant IFNGR1s (29) (data not shown). The functions of the other phospho-tyrosine residues of IFNGR1, Tyr440, appears to be different: the hybrid murine fibroblast cell line SCC16-5, containing a single copy of human chromosome 21 encoding human IFNGR2, was used initially to characterize human IFNGR1 (49, 50). The expression of various mutants of IFNGR1 in these cells revealed that Tyr<sup>440</sup> is required for STAT1 activation (46), the antiviral response (Fig. 3c), and the transcription of ISGs in reconstituted IFNGR1-null MEFs. It is interesting that the requirement for the corresponding tyrosine residue of human IFNGR1, Tyr<sup>440</sup>, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited. The hybrid murine fibroblast cell line SCC16-5, containing a single copy of human chromosome 21 encoding human IFNGR2, was used initially to characterize human IFNGR1 (49, 50). The expression of various mutants of IFNGR1 in these cells revealed that Tyr<sup>440</sup> is required for STAT1 activation (46), the antiviral response (Fig. 3c), and the transcription of ISGs in reconstituted IFNGR1-null MEFs. It is interesting that the requirement for the corresponding tyrosine residue of human IFNGR1, Tyr<sup>440</sup>, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited. The hybrid murine fibroblast cell line SCC16-5, containing a single copy of human chromosome 21 encoding human IFNGR2, was used initially to characterize human IFNGR1 (49, 50). The expression of various mutants of IFNGR1 in these cells revealed that Tyr<sup>440</sup> is required for STAT1 activation (46), the antiviral response (Fig. 3c), and the transcription of ISGs in reconstituted IFNGR1-null MEFs. It is interesting that the requirement for the corresponding tyrosine residue of human IFNGR1, Tyr<sup>440</sup>, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited.

**Interaction of SOCS-1 and IFNGR1—**Analyses of the mechanisms of the inhibitory effects of SOCS-1 have focused on the ability of SOCS-1 to bind to the active loop of JAKs, and interaction of SOCS-1 with the interleukin-2 receptor β chain.

**DISCUSSION**

**Contribution of Specific Tyrosine Residues of IFNGR1 to IFNγ-dependent Signaling—**In response to IFNγ, the phospho-tyrosine residues of IFNGR1 have not been investigated until now. Here we show that an important function of one of these residues is attenuation of IFNγ-induced antiviral responses, which are stronger in IFNGR1-null MEFs expressing mutant IFNGR1s (29) (data not shown). The functions of the other phospho-tyrosine residues of IFNGR1, Tyr440, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited. It is interesting that the requirement for the corresponding tyrosine residue of human IFNGR1, Tyr440, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited. The hybrid murine fibroblast cell line SCC16-5, containing a single copy of human chromosome 21 encoding human IFNGR2, was used initially to characterize human IFNGR1 (49, 50). The expression of various mutants of IFNGR1 in these cells revealed that Tyr440 is required for STAT1 activation (46), the antiviral response (Fig. 3c), and the transcription of ISGs in reconstituted IFNGR1-null MEFs. It is interesting that the requirement for the corresponding tyrosine residue of human IFNGR1, Tyr440, appears to be different: in primary human fibroblasts lacking IFNGR1 and reconstituted with the Y440F mutant, the expression of ISGs in response to IFNγ was substantial, although the antiviral effect was inhibited.

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<sup>3</sup> Y. Qing and G. R. Stark, unpublished data.

<sup>4</sup> A. P. Costa-Pereira, D. Watling, and I. M. Kerr, unpublished data.
has been shown not to be required for inhibitory effects (52). Our results show, that in IFNγ-dependent signaling, constitutive overexpression of SOCS-1 partially inhibited JAK2 phosphorylation (Fig. 4b), and this inhibition was observed only in cells retaining Tyr441 of IFNGR1. We were also able to show that SOCS-1 binds to IFNGR1 at Tyr441 in a ligand-dependent manner, presumably through an interaction between its SH2 domain and phospho-tyrosine 441, because tyrosine to phenylalanine mutation of Tyr441 abolishes the interaction (Fig. 5). These results, together with others in the literature, suggest that SOCS-1, like SOCS-3 (the most homologous member of the SOCS family) (53), is likely to inhibit cytokine-dependent signaling though its interaction with a receptor (7, 31, 34), although SOCS-1 does interact with and inhibit JAKs in vitro (14, 48).

How SOCS-1 Inhibits IFNγ-dependent Signaling—Studies in vitro have shown that SOCS-1 inhibits the kinase activity of JAK2, probably by binding to the active site loop (14, 48), and this inhibition was observed only in cells expressing wild-type IFNGR1 or the 2F/441Y/419Y mutant and that mutation of Tyr441 completely abrogates the IFNγ-dependent degradation (7). Our results show that SOCS-1 and Tyr441 of IFNGR1, IFNγ-dependent signaling was prolonged, revealing that the interaction of SOCS-1 and Tyr441 of IFNGR1 is required for negative regulation. A likely scenario is that, in response to IFNγ, SOCS-1 expression is induced, and SOCS-1 is recruited to IFNGR1 through phospho-tyrosine 441, which brings it close enough to JAK2 to enable it to bind to the active site loop, thus inhibiting the kinase activity and possibly also catalyzing proteasome-mediated degradation of JAK2, leading to negative feedback of IFNγ-dependent signaling. However, the relatively small effect on JAK2 phosphorylation contrasts with the dramatic effect on STAT1 phosphorylation (Fig. 4).

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