Identification of CXCL11 as a STAT3-Dependent Gene Induced by IFN

Chuan He Yang, Lai Wei, Susan R. Pfeffer, Ziyun Du, Aruna Murti, William J. Valentine, Yi Zheng and Lawrence M. Pfeffer

J Immunol 2007; 178:986-992; doi: 10.4049/jimmunol.178.2.986
http://www.jimmunol.org/content/178/2/986

References
This article cites 39 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/178/2/986.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Identification of CXCL11 as a STAT3-Dependent Gene Induced by IFN

Chuan He Yang,2* Lai Wei,2* Susan R. Pfeffer,* Ziyun Du,* Aruna Murti,* William J. Valentine,* Yi Zheng,† and Lawrence M. Pfeffer3*

IFNs selectively regulate gene expression through several signaling pathways. The present study explored the involvement of STAT3 in the IFN-induced expression of the gene encoding the CXCL11 chemokine. The CXCL11 gene was induced in IFN-sensitive Daudi cells, but not in an IFN-resistant DRST3 subline with a defective STAT3 signaling pathway. Although the IFN-stimulated gene ISG15 was induced to a similar extent in Daudi and DRST3 cells, expression of wild-type STAT3 in DRST3 cells restored the IFN inducibility of CXCL11. Reconstitution of STAT3 knockout mouse embryonic fibroblasts with wild-type STAT3, or STAT3 with the canonical STAT3 dimerization site at Y705 mutated, restored IFN inducibility of the CXCL11 gene. These data indicate that CXCL11 gene induction by IFN is STAT3 dependent, but that phosphorylation of Y705 of STAT3 is not required. Chromatin immunoprecipitation assays demonstrated that IFN treatment of Daudi and DRST3 cells induced STAT3 binding to the CXCL11 promoter. Chromatin immunoprecipitation assays also revealed that NF-κB family member p65 and IFN regulatory factor (IRF)1 were bound to CXCL11 promoter upon IFN treatment of Daudi cells. In contrast, IFN induced the binding of p50 and IRF2 to the CXCL11 promoter in DRST3 cells. The profile of promoter binding was indistinguishable in IFN-sensitive Daudi cells and DRST3 cells reconstituted with wild-type STAT3. Thus, STAT3 also plays a role in the recruitment of the transcriptional activators p65 and IRF1, and the displacement of the transcriptional repressors p50 and IRF2 from the CXCL11 promoter also appears to regulate the induction of CXCL11 gene transcription. The Journal of Immunology, 2007, 178: 986–992.

Although originally identified by their antiviral activity, IFNs are diverse multifunctional cytokines that also inhibit cell proliferation, regulate cell differentiation, regulate apoptosis (programmed cell death), and modulate activities of the immune system. Furthermore, IFNs have clinical utility in diseases of diverse pathogenesis and manifestations including hairy cell leukemia, Kaposi’s sarcoma, laryngeal and genital papillomas, chronic viral hepatitis, and multiple sclerosis. The type I IFNs, consisting of IFN-α, IFN-β, and IFN-ω (IFN-αβ), bind to a multisubunit cell surface receptor complex that is distinct from the receptor for type II IFN, IFN-γ. IFN-αβ elicits diverse biological effects by altering the pattern of gene expression through the activation of the JAK1 and TYK2 nonreceptor protein tyrosine kinases, which mediate the tyrosine phosphorylation of several STAT proteins. STAT1 and STAT2 play crucial roles in the transcriptional response to IFN-αβ and in the induction of antiviral activity (1, 2). In the classical JAK-STAT signaling pathway, these phosphorylated STAT proteins form a complex with IFN regulatory factor (IRF)4,9, translocate into the nucleus, and bind to the conserved IFN stimulation response element (ISRE) within the promoters of IFN-stimulated genes (ISGs).

The importance of STAT3 in IFN-αβ action has been defined by characterizing the IFN response pathway in Daudi lymphoblastoid cells and in an IFN-resistant subclone (3–5). However, the role of STAT3 in IFN-induced gene expression is unknown. The general dogma is that a single tyrosine is phosphorylated in STATs upon cytokine stimulation (Y705 in STAT3), which results in STAT dimerization and subsequent binding to the promoter of STAT target genes (6). However, 21 of 22 potential tyrosine-based motifs are perfectly conserved in human, murine, and rat STAT3 homologs. Although Daudi cells are extremely sensitive to the antiproliferative and antiviral actions of IFN, IFN-resistant Daudi cells are relatively nonresponsive and stably maintain their resistance in the absence of IFN addition (7–14). IFN-sensitive and IFN-resistant cell lines have similar numbers of IFN receptors, and activate the JAK-STAT signaling pathway as defined by IFN-dependent tyrosine phosphorylation of JAK1, TYK2, STAT1, and STAT2 and the induction of ISG transcription. Although IFN-resistant Daudi cells have detectable levels of STAT3, these cells exhibit a defective STAT3-dependent IFN signaling pathway, which can be rescued by stable expression of STAT3. Hence Daudi cells are designated DRST3 (for Daudi cells that are IFN-resistant to the antiviral and antiproliferative effects and exhibit a defective STAT3-dependent signaling pathway).

The present study explored the involvement of STAT3 in the expression of the IFN-induced gene CXCL11, which encodes the

*Department of Pathology and Laboratory Medicine, University of Tennessee Health Science Center and University of Tennessee Cancer Institute, Memphis, TN 38163; and 1Division of Experimental Hematology, Children’s Hospital Research Foundation, University of Cincinnati, Cincinnati, OH 45229

Received for publication May 19, 2006. Accepted for publication October 25, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by grant CA73753 from the National Institutes of Health (to L.M.P.) and by funds from the Muirhead Chair Endowment at the University of Tennessee Health Science Center and University of Tennessee Cancer Institute, Memphis, TN 38163; and 2Division of Experimental Hematology, Children’s Hospital Research Foundation, University of Cincinnati, Cincinnati, OH 45229

2 C.H.Y. and L.W. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Lawrence M. Pfeffer, Department of Pathology and Laboratory Medicine, University of Tennessee Health Science Center, 930 Madison Avenue, Room 530, Memphis, TN 38163. E-mail address: LPfeffer@UTMEM.edu

www.jimmunol.org

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

*Abbreviations used in this paper: IRF, IFN regulatory factor; ChIP, chromatin immunoprecipitation; ISRE, IFN stimulation response element; SIE, c-sis inducible element; ISG, IFN-stimulated gene; KO, knockout; MEF, mouse embryonic fibroblast; WT, wild type.*
Chemokine also called β-R1 and I-TAC. Our data indicate that CXCL11 gene induction by IFN is STAT3 dependent, and that phosphorylation of the Y705 (the canonical dimerization site) of STAT3 is not required for the IFN inducibility of CXCL11 gene expression. Other events such as the recruitment of the transcriptional activators p65, which is a NF-kB family member, and IRF1 and the displacement of the transcriptional repressors p50 and IRF2 from the CXCL11 promoter are also STAT3 dependent and regulate the IFN induction of CXCL11 gene transcription.

Materials and Methods

Biological reagents and cell culture

InterMune and Biogen-Idec provided recombinant human IFNαCon1 and rat IFN-β, respectively. IFN biological activity was expressed in international reference units per millilitre as assayed by protection against the cytopathic effect of vesicular stomatitis virus on fibroblasts, using the appropriate National Institutes of Health reference standard. Abs directed against the following proteins were used: STAT1, phospho-STAT1, STAT2, IRF1, IRF2, p65, and p50 from Santa Cruz Biotechnology; STAT3 from BD Biosciences; and Y705 phosphorylated of STAT3 from Upstate Biotechnology. IFN-sensitive Daudi cells and the IFN-resistant Daudi (DRST3) cells were maintained at 2–10 × 10⁵ cells/ml in RPMI 1640 containing 10% defined calf serum (HyClone). STAT3-knockout (STAT3-KO) mouse embryonic fibroblasts (MEFs) and reconstituted MEFs were generated as previously described (15) and plated at 1 × 10⁵ cells/cm² every 3 days in DMEM supplemented with 10% defined calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin.

Transfection conditions and constructs

To determine the role of STAT3 in ISG expression, cells were transfected with expression plasmids. Transient and stable transfection of cells (10⁵) was accomplished by electroporation (capacitance 300 μF, 250 V) with 50 μg of salmon sperm DNA and 20 μg of plasmid DNA for each sample. STAT3 was cloned in the pEF expression vector, which provides a c-myc epitope tag at the COOH terminus of the protein (5).

Immunoprecipitations and immunoblot analysis

For immunoprecipitation studies, transiently transfected cells were treated with IFN (1000 U/ml) at 37°C for the indicated time periods and then washed with ice-cold PBS and lysed 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 Na₂VO₄, 1 mM PMSF, 5 µg/ml soybean trypsin inhibitor, 5 µg/ml leupeptin, and 1.75 µg/ml benzamidine. Samples were clarified by centrifugation at 12,000 × g for 15 min at 4°C and were immunoprecipitated with anti-myc (Santa Cruz Biotechnology) overnight at 4°C. Immune complexes were collected using protein A-Sepharose beads (Pharmacia) and eluted in sample buffer. Samples were separated on SDS-7.5% PAGE, transferred to polyvinylidene difluoride membranes (Millipore) and probed with anti-STAT1 or anti-STAT3 (dilution 1/1000), followed by anti-mouse IgG coupled with HRP (Santa Cruz Biotechnology). Blots were developed using ECL (Pierce).

Nuclear extracts and DNA binding activity assays

Nuclei isolated from control and IFN-treated cells were extracted with buffer (20 mM Tris-HCl (pH 7.85), 250 mM sucrose, 0.4 M KCl, 1.1 mM MgCl₂, 5 mM 2-ME, 1 mM NaF, 1 mM Na₂VO₄, 1 mM PMSF, 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 (5). The nuclear extracts were incubated with a ³²P-labeled probe for the high affinity c-κB inducible element (SIE) in the c-fos gene (5'-AGCTTTGATTTCCGGCAATTC-3') and subjected to EMSA (4). To define the presence of specific STAT proteins in DNA-protein complexes, nuclear extracts were preincubated with a 1:50 dilution of anti-STAT Abs at 25°C for 20 min before EMSA. Gels were quantitated by PhosphorImaging autoradiography.

Quantitative real-time PCR

Total RNA was isolated from untreated and IFN-treated cells using TRIzol reagent (Invitrogen Life Technologies). Quantitative RT-PCR was performed on an iCycler (Bio-Rad) using the AccessQuick RT-PCR system (Promega), and SYBR Green I (Molecular Probes) according to the manufacturer’s instructions. The following forward and reverse primers used were: human CXCL11, 5'-ATGAGTTGTAAGGCGATGCG-3' (forward), 5'-TCACGTGTTCCTACCCAGG-3' (reverse); human ISG15, 5'-GGTCCA GTGTGCTGAAAGGC-3' (forward), 5'-TTGTGTCGGAGATCTGCG-3' (reverse); human β-actin, 5'-AGAAGGAGATACGTCCCTG-3' (forward), 5'-CACATCTCGTGAAGGTTGGA-3' (reverse); mouse CXCL11, 5'-AGGA GGTCAAGCCATAGC-3' (forward), 5'-CGACTCTGCGCATTTTGGAGC-3' (reverse); and mouse β-actin, 5'-AAGGAGATTCTGCTGGC-3' (forward), 5'-CACTACTGCTGGAGGTTGAC-3' (reverse).

Reverse transcription was performed at 48°C for 45 min and RT-PCR cycling parameters were as follows: denaturation at 95°C for 2 min, amplification at 94°C for 30 s, and 60°C for 30 s for 35 cycles. The product size was initially monitored by agarose gel electrophoresis, and melting curves were analyzed to control for specificity of PCR. The data on IFN-induced genes were normalized to the expression of the housekeeping gene β-actin. The relative units were calculated from a standard curve, plotting the fluorescent signal against the PCR cycle number at which the measured intensity reaches a fixed value (with a 10-fold increment equivalent to ~3.1 cycles).

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed using the Chip-IT kit (Active Motif) according to the manufacturer’s instructions with the average size of sheared fragments ~200 bp. The forward and reverse primers used were human CXCL11 5'-GGTTTCACATGCTTTCTC-3' (forward), 5'-TTTCCCCCTTGGACTG-3' (reverse).

Results

Induction of CXCL11 by IFN is dependent on STAT3

To determine the effect of STAT3 on IFN-induced CXCL11 expression, quantitative real-time PCR assays were performed for CXCL11 and ISG15 using RNA from Daudi cells and DRST3 cells that were treated with IFN for 6 h. The expression of ISG15 was determined because the IFN induction of this gene is through the classical JAK-STAT pathway involving the binding of STAT1-STAT2-IRF9 complexes to the ISRE. As shown in Fig. 1, IFN treatment induced a dose-dependent increased expression of CXCL11 (Fig. 1A) and ISG15 (Fig. 1B) in IFN-sensitive Daudi cells. In contrast, IFN treatment induced expression of ISG15, but not CXCL11 in IFN-resistant DRST3 cells. CXCL11 gene expression was not observed in DRST3 cells up to 24 h after IFN addition and could not be induced at IFN concentrations as high as 10,000 U/ml (data not shown).

DRST3 cells have a defective STAT3-dependent signaling pathway, which can be rescued by expression of wild-type (WT) STAT3 in these cells (4). To determine whether STAT3 can rescue the IFN-induced expression of the CXCL11, RNA was isolated from DRST3 cells stably expressing WT STAT3 (STAT3-DRST3 cells) after IFN treatment and subjected to quantitative real-time PCR assays for CXCL11 and ISG15. As shown in Fig. 1A, STAT3 expression was required for the induction of CXCL11 gene expression by IFN. In contrast, expression of STAT3 in these IFN-resistant cells had no effect on the induction of ISG15 by IFN. Taken together these results suggested that IFN-induced expression of CXCL11 is STAT3 dependent, but expression of ISG15 is STAT3 independent.

To further characterize the induction of CXCL11 expression by IFN, we examined the effect of inhibitors of RNA synthesis and histone deacetylation on IFN-induced gene expression. Inhibition of RNA synthesis by treatment with actinomycin D blocked the induction of ISG15 and CXCL11 by IFN (data not shown), demonstrating that the induction of ISG15 and CXCL11 by IFN reflects transcription activation rather than an effect on RNA stability. Moreover, as shown in Fig. 1C treatment of Daudi cells with the histone deacetylase inhibitor trichostatin A did not increase the basal expression of ISG15 or CXCL11, but did block the IFN-induced expression of these genes. Our results are consistent with previous finding that the inhibition of histone deacetylase function.

The Journal of Immunology
led to impairment of IFN-induced ISG expression, with little effect on basal ISG expression (16).

Tyrosine phosphorylation, dimerization, and binding of STAT3 to the CXCL11 promoter

Phosphorylation of STAT proteins at a critical tyrosine residue is necessary for their appropriate transactivation function as transcription factors (2). For STAT3, phosphorylation at Y705 has been shown previously to be required for its dimerization and function. To determine the phosphorylation of STAT3 at this critical tyrosine residue, we immunoprecipitated STAT3 from cell lysates prepared from Daudi cells and DRST3 cells after IFN treatment, and then performed Western blot analysis with Y705 phospho-specific Ab. As shown in Fig. 2A, STAT3 underwent phosphorylation at Y705 in both IFN-sensitive Daudi cells and IFN-resistant DRST3 cells within 10 min of IFN addition with significant levels of phosphorylation observed at 30 min after IFN addition. Consistent with previous studies relatively low levels of STAT3 tyrosine phosphorylation were detected in IFN-treated DRST3 cells as compared with Daudi cells with a general anti-phosphotyrosine Ab (4). Blotting with anti-STAT3 verified that equivalent amounts of STAT3 were expressed in Daudi and DRST3 cells. Thus, STAT3 undergoes phosphorylation of Y705 in response to IFN in IFN-resistant DRST3 cells.

To assess for the role of Y705 in the formation of STAT3 complexes, DRST3 cells were stably transfected with either WT-STAT3 or a mutant form of STAT3 (F705-STAT3), in which tyrosine residue 705 was mutated to a phenylalanine, and STAT3 immunoprecipitates were probed with anti-STAT1. As shown in Fig. 2B, IFN treatment of IFN-resistant DRST3 cells did not induce formation of STAT1-STAT3 complexes. However, IFN

FIGURE 2. IFN induces Y705 phosphorylation in DRST3 cells and the formation of STAT1/STAT3 dimers. A, Lysates prepared from Daudi and DRST3 cells at the indicated time after IFN-α addition (1000 U/ml) were immunoprecipitated with anti-STAT3. The proteins were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes and probed with anti-STAT3, anti-Y705-STAT3, and anti-phosphotyrosine (clone 4G10; Upstate Biotechnology). B, Lysates prepared from control and IFN-α-treated (1000 U/ml, 15 min) DRST3 cells transfected with either WT STAT3, F705 mutant STAT3, or empty vector (EV) were immunoprecipitated with anti-myc. The proteins were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and probed with either anti-STAT1 or anti-STAT3. Blots were visualized by ECL (Pierce). C, Nuclear extracts from control or IFN-α-treated (1000 U/ml, 15 min) DRST3 cells transfected with WT STAT3, F705 mutant STAT3, or empty vector (EV) were incubated with a 32P-labeled SIE probe. To define specific STAT proteins, extracts were preincubated with anti-STAT1 or anti-STAT3 Abs before EMSA. Results from one of two experiments are shown.
treatment of DRST3 cells expressing WT-STAT3 induced the formation of STAT1/STAT3 dimers. In DRST3 cells expressing the F705-STAT3 mutant, although similar levels of STAT3 were expressed, STAT3 did not form a complex with STAT1. These data indicate that IFN-induced phosphorylation of Y705 is necessary for the formation of STAT1-STAT3 complexes.

The activation of STAT3 can also be demonstrated by its presence in DNA-protein complexes using an oligonucleotide SIE probe (3). This finding led us next to investigate the IFN-dependent activation of STAT3-containing binding complexes. As illustrated in Fig. 2C and consistent with our previous findings (5), IFN only induces the formation of STAT1/STAT1 homodimers in IFN-resistant DRST3 cells, which are supershifted by anti-STAT1 but not by anti-STAT3. However, expression of WT-STAT3 in the IFN-treated DRST3 cells results in the induction by IFN of two additional STAT3-dependent SIE binding complexes (STAT1/STAT3 heterodimers and STAT3/STAT3 homodimers) as demonstrated by supershift assays with anti-STAT1 and anti-STAT3. All three SIE binding complexes are also formed in IFN-sensitive Daudi cells (5). In DRST3 cells expressing the F705-STAT3 mutant, IFN did not induce either of the STAT3-dependent DNA binding complexes, although similar levels of STAT3 were expressed. These data indicate that IFN-induced phosphorylation of Y705 is necessary for the formation of STAT3-dependent DNA binding complexes.

**Phosphorylation of Y705 of STAT3 is not required for IFN-induced CXCL11 expression**

To further characterize the role of STAT3 as well as the tyrosine phosphorylation of Y705 of STAT3 in CXCL11 induction by IFN, MEFs with a germline deletion of STAT3 were examined (15). These STAT3-KO MEFs were reconstituted with WT-STAT3 or the F705-STAT3 mutant, and quantitative real-time PCR assays were performed for CXCL11. As shown in Fig. 3A, CXCL11 reached an induction level of \( \sim 150 \)-fold in STAT3-KO MEFs stably expressing either WT-STAT3 or F705-STAT3 after 6 h of IFN treatment, but was only slightly induced in STAT3-KO MEFs. Similar expression levels of IFN-induced CXCL11 were observed with WT MEFs that have STAT3 protein levels comparable to STAT3-KO MEFs reconstituted with STAT3. These results indicate that although STAT3 is required for IFN-induced expression of the CXCL11 gene, tyrosine phosphorylation on Y705 of STAT3 is not required for IFN-induced CXCL11 gene expression. This result is surprising in light of the critical role Y705 of STAT3 apparently plays in tumorigenesis and JAK-STAT signal transduction by cytokines.

IFN-mediated signal transduction involves not only JAK-mediated tyrosine phosphorylation, but also activation of a PI3K-Akt serine phosphorylation pathway (3, 17, 18). To characterize the role of protein kinases in IFN induction of CXCL11 gene expression, MEFs stably expressing the F705-STAT3 mutant were treated with genistein (tyrosine kinase inhibitor), LY294002 (PI3K inhibitor), or staurosporin, which blocks IFN-induced biological events mediated by protein kinase C and by tyrosine kinases (8, 19, 20). As shown in Fig. 3B treatment with genistein, staurosporin, or LY294002 blocked induction of CXCL11 expression by IFN. The data indicate that both JAK-mediated tyrosine phosphorylation, and activation of a PI3K-Akt serine phosphorylation pathway are involved in IFN-induced CXCL11 gene expression.

**The binding of transcription factors to the CXCL11 promoter**

To investigate whether STAT3 directly bound to the CXCL11 promoter, ChIP assays were performed. As shown in Fig. 4, IFN treatment induced the binding of STAT3 to the CXCL11 promoter in Daudi cells as early as 1 h of IFN treatment and STAT3 remained bound to the CXCL11 promoter up to 3 h of IFN treatment. Surprisingly, STAT3 was also recruited to CXCL11 promoter after IFN treatment in DRST3 cells. Moreover, ChIP assays performed with Y705 phospho-specific Ab showed that upon IFN treatment Y705 phosphorylated STAT3 was recruited onto CXCL11 promoter in both Daudi and DRST3 cells. These results indicated that recruitment of STAT3 to the CXCL11 promoter may be important for IFN-induced CXCL11 expression, but this event alone may not be sufficient for gene expression.

Because Y705 of STAT3 is not required for IFN-induced CXCL11 expression, we performed ChIP assays to profile binding of other potential transcription factors to the CXCL11 promoter. Previous studies showed that IFN treatment of Daudi cells induced the formation of multiple STAT dimers, which include various

---

**FIGURE 3.** Phosphorylation of Y705 of STAT3 is not required for IFN-induced CXCL11 expression and the effect of protein kinase inhibitors on IFN-induced CXCL11 expression. **A**, Real-time PCR for CXCL11 gene expression was performed on cDNAs prepared from WT MEFs, STAT3-KO cells, or STAT3-KO MEFs reconstituted with WT STAT3 or the F705-STAT3 mutant at varying times after IFN addition (1000 U/ml). **B**, Real-time PCR for CXCL11 gene expression was performed on cDNAs prepared from STAT3-KO MEFs reconstituted with the F705-STAT3 mutant, which were pretreated with DMSO as vehicle, the tyrosine kinase inhibitor genistein (100 µM), or the general protein kinase inhibitor staurosporin (50 nM) for 1 h before IFN addition (1000 IU/ml, 5 h). Gene expression was normalized to actin expression. Data are shown as fold induction relative to untreated cells and are mean ± SE (n = 3 experiments).
FIGURE 4. The effects of IFN on the binding of STAT, NF-κB, and IRF proteins to the CXCL11 promoter. ChIP assays were performed on extracts from control and IFN-treated Daudi cells, DRST3 cells, and DRST3 cells expressing WT STAT3 using the indicated Abs for precipitation and primers that targeted the CXCL11 promoter as described in Materials and Methods. Similar results were obtained in at least three independent experiments.

Discussion

STAT3, the transcription factor for acute phase response genes, is activated by a wide variety of cytokines suggesting that it may integrate diverse signals into common transcriptional responses (5, 22, 23). A critical role of STAT3 in cellular physiology is implicated by the finding that the KO of the STAT3 gene in mice leads to early embryonic lethality, and STAT3-deficient cell lines could not be isolated (24). Moreover, STAT3 apparently plays an important role in tumorigenesis by regulating cell cycle progression, apoptosis, angiogenesis, invasion and metastasis, and evasion of immune surveillance (25–28). A tyrosine-based YXXQ motif in the cytosolic tails of type I IFN receptor 1 (IFNAR1) α chain of the IFN-αβ receptor and in the shared signal-transducing gp130 chain of the IL-6R family is required for cytokine-dependent STAT3 tyrosine phosphorylation and dimerization (12, 29–31). Although the role that STAT1/STAT2 dimers play in ISG activation is well understood, it is unknown how STAT3-containing dimers form and what is their role in IFN-responsive gene expression.

We examined the role of STAT3 in the regulation of IFN-responsive gene expression. STATs, like many cytoplasmic effectors, contain tyrosine-based motifs as well as Src homology 2 domains (32) that bind tyrosine-based motifs. The general dogma in the cytokine field is that a single tyrosine is phosphorylated in STATs upon cytokine stimulation (Y705 in STAT3), followed by tyrosine phosphorylation and dimerization (12, 29 –31). Although the pattern of transcription factor binding was indistinguishable from that in IFN-sensitive Daudi cells, i.e., the IFN-induced loss of p50 and IRF2 promoter binding, and the IFN-induced recruitment of p65 and IRF1 binding to the CXCL11 promoter.

We have recently shown that NF-κB proteins play a key regulatory role in IFN-induced gene expression (21). In Daudi cells, the p50 NF-κB subunit was found basally bound to the CXCL11 promoter and by 1 h of IFN treatment the p50 subunit of NF-κB was no longer found associated with the CXCL11 promoter. In contrast, the p65 subunit of NF-κB was not basally promoter-bound but its binding was induced by 1 h after IFN addition. In DRST3 cells, although p50 was not basally bound to the CXCL11 promoter, IFN induced p50 binding within 1 h after addition, and p65 was neither basally promoter-bound nor was its binding IFN induced. Moreover, IFN induced the binding of the transcriptional activator IRF1 to the CXCL11 promoter in Daudi cells. In contrast, in DRST3 cells IFN induced the binding of another IRF family member, IRF2, which is a transcriptional repressor. These results indicate that the defective STAT3 signaling pathway in DRST3 cells affects the binding of NF-κB and IRF proteins, which cooperate in the transcription regulation of CXCL11. To rescue the defective STAT3 signaling pathway in DRST3 cells, we examined the effect of expression of WT-STAT3 on the binding of NF-κB and IRF proteins to the CXCL11 promoter. As shown in Fig. 4, expression of WT-STAT3 in DRST3 cells rescued the recruitment of NF-κB and IRF proteins to the CXCL11 promoter by IFN so that the pattern of transcription factor binding was indistinguishable from that in IFN-sensitive Daudi cells, i.e., the IFN-induced loss of p50 and IRF2 promoter binding, and the IFN-induced recruitment of p65 and IRF1 binding to the CXCL11 promoter. The effect of expression of WT-STAT3 on the binding of NF-κB, and IRF proteins to the CXCL11 promoter rescued the recruitment of NF-κB and IRF proteins to the CXCL11 promoter by IFN so that expression of WT-STAT3 in DRST3 cells rescued the recruitment of NF-κB and IRF proteins to the CXCL11 promoter by IFN so that the pattern of transcription factor binding was indistinguishable from that in IFN-sensitive Daudi cells, i.e., the IFN-induced loss of p50 and IRF2 promoter binding, and the IFN-induced recruitment of p65 and IRF1 binding to the CXCL11 promoter.

Discussion

STAT3, the transcription factor for acute phase response genes, is activated by a wide variety of cytokines suggesting that it may integrate diverse signals into common transcriptional responses (5, 22, 23). A critical role of STAT3 in cellular physiology is implicated by the finding that the KO of the STAT3 gene in mice leads to early embryonic lethality, and STAT3-deficient cell lines could not be isolated (24). Moreover, STAT3 apparently plays an important role in tumorigenesis by regulating cell cycle progression, apoptosis, angiogenesis, invasion and metastasis, and evasion of immune surveillance (25–28). A tyrosine-based YXXQ motif in the cytosolic tails of type I IFN receptor 1 (IFNAR1) α chain of the IFN-αβ receptor and in the shared signal-transducing gp130 chain of the IL-6R family is required for cytokine-dependent STAT3 tyrosine phosphorylation and dimerization (12, 29–31). Although the role that STAT1/STAT2 dimers play in ISG activation is well understood, it is unknown how STAT3-containing dimers form and what is their role in IFN-responsive gene expression.

We examined the role of STAT3 in the regulation of IFN-responsive gene expression. STATs, like many cytoplasmic effectors, contain tyrosine-based motifs as well as Src homology 2 domains (32) that bind tyrosine-based motifs. The general dogma in the cytokine field is that a single tyrosine is phosphorylated in STATs upon cytokine stimulation (Y705 in STAT3), followed by tyrosine phosphorylation and dimerization (12, 29 –31). Although the pattern of transcription factor binding was indistinguishable from that in IFN-sensitive Daudi cells, i.e., the IFN-induced loss of p50 and IRF2 promoter binding, and the IFN-induced recruitment of p65 and IRF1 binding to the CXCL11 promoter.

We have recently shown that NF-κB proteins play a key regulatory role in IFN-induced gene expression (21). In Daudi cells, the p50 NF-κB subunit was found basally bound to the CXCL11 promoter and by 1 h of IFN treatment the p50 subunit of NF-κB was no longer found associated with the CXCL11 promoter. In contrast, the p65 subunit of NF-κB was not basally promoter-bound but its binding was induced by 1 h after IFN addition. In DRST3 cells, although p50 was not basally bound to the CXCL11 promoter, IFN induced p50 binding within 1 h after addition, and p65 was neither basally promoter-bound nor was its binding IFN induced. Moreover, IFN induced the binding of the transcriptional activator IRF1 to the CXCL11 promoter in Daudi cells. In contrast, in DRST3 cells IFN induced the binding of another IRF family member, IRF2, which is a transcriptional repressor. These results indicate that the defective STAT3 signaling pathway in DRST3 cells affects the binding of NF-κB and IRF proteins, which cooperate in the transcription regulation of CXCL11. To rescue the defective STAT3 signaling pathway in DRST3 cells, we examined the effect of expression of WT-STAT3 on the binding of NF-κB and IRF proteins to the CXCL11 promoter. As shown in Fig. 4, expression of WT-STAT3 in DRST3 cells rescued the recruitment of NF-κB and IRF proteins to the CXCL11 promoter by IFN so that the pattern of transcription factor binding was indistinguishable from that in IFN-sensitive Daudi cells, i.e., the IFN-induced loss of p50 and IRF2 promoter binding, and the IFN-induced recruitment of p65 and IRF1 binding to the CXCL11 promoter.

Discussion

STAT3, the transcription factor for acute phase response genes, is activated by a wide variety of cytokines suggesting that it may integrate diverse signals into common transcriptional responses (5, 22, 23). A critical role of STAT3 in cellular physiology is implicated by the finding that the KO of the STAT3 gene in mice leads to early embryonic lethality, and STAT3-deficient cell lines could not be isolated (24). Moreover, STAT3 apparently plays an important role in tumorigenesis by regulating cell cycle progression, apoptosis, angiogenesis, invasion and metastasis, and evasion of immune surveillance (25–28). A tyrosine-based YXXQ motif in the cytosolic tails of type I IFN receptor 1 (IFNAR1) α chain of the IFN-αβ receptor and in the shared signal-transducing gp130 chain of the IL-6R family is required for cytokine-dependent STAT3 tyrosine phosphorylation and dimerization (12, 29–31). Although the role that STAT1/STAT2 dimers play in ISG activation is well understood, it is unknown how STAT3-containing dimers form and what is their role in IFN-responsive gene expression.

We examined the role of STAT3 in the regulation of IFN-responsive gene expression. STATs, like many cytoplasmic effectors, contain tyrosine-based motifs as well as Src homology 2 domains (32) that bind tyrosine-based motifs. The general dogma in the cytokine field is that a single tyrosine is phosphorylated in STATs upon cytokine stimulation (Y705 in STAT3), followed by STAT dimerization and the transcriptional activation of selective gene expression (6). We found that STAT3 is required for IFN-induced expression of CXCL11 expression in both STAT3-KO MEFs and in STAT3-defective Daudi human lymphoblastoid cells. However, although phosphorylation of the Y705 residue of STAT3 is critical for the formation of STAT1/STAT3 heterodimers as detected by coimmunoprecipitation or EMSA, this event is not required for IFN-induced CXCL11 gene expression. Thus, in future studies it will be important to identify and characterize other STAT3-dependent genes induced by IFN that do not require Y705 phosphorylation of STAT3. The finding that Y705 phosphorylation is not required for IFN regulation of CXCL11 gene expression
is somewhat surprising in light of the findings that constitutive Y705 phosphorylation of STAT3 occurs in a number of human cancers and is considered a marker of activated STAT3. We hypothesize that phosphorylation of different tyrosine and/or serine residues of STAT3 may result in differential regulation of STAT3 target genes. For example, the IFN-induced phosphorylation of a Y657 KIM motif of STAT3 appears to act as a docking site for the lipid/serine kinase PI3K (3). In the present study, we show that pharmacological inhibitors of PI3K and tyrosine kinases inhibit IFN induction of CXCL11 gene expression, which is in agreement with previous finding for a role of JAK-STAT and PI3K signaling in the activation of a CXCL11 promoter-reporter construct by IFN (33).

The CXCL11 gene has been shown to be selectively induced by IFN-β relative to IFN-α in human cells (34). Consistent with these results we show that in murine fibroblasts IFN-β results in a higher CXCL11 gene induction than does IFN-α in human lymphoblastoid cells. Moreover, previous studies established that the activation of the JAK-STAT signaling pathway as well as the NF-κB pathway is necessary for CXCL11 gene induction (35). Site-directed mutagenesis of the ISRE or the NF-κB binding site in a promoter-reporter construct of the CXCL11 gene abrogated IFN induction of the reporter construct. Our studies show that the “classical” ISRE binding protein complex composed of STAT1-STAT2 does not bind to the CXCL11 promoter, although both STAT1 and STAT2 are activated in Daudi cells, as demonstrated by the formation of an ISRE complex using a consensus ISRE oligonucleotide probe as well as by their tyrosine phosphorylation upon IFN treatment (4). In contrast, we show that STAT3 and STAT1 bind to the CXCL11 promoter, as well as the ISRE binding proteins IRF1 and IRF2.

In addition, we show that NF-κB proteins regulate the IFN-induced expression of CXCL11 gene. The NF-κB proteins p50, p52, p65 RelB, and c-Rel are transcription factors that regulate the expression of genes involved in the immune response, inflammation, and cell survival. These proteins dimerize and bind to the promoter elements of genes to regulate their expression. In a series of recent studies we show that different NF-κB proteins bind to the promoters of ISGs to regulate their expression (21). We previously showed that STAT3 functions as an adapter protein to couple signaling cascades such as PI3K/Akt to IFN signaling (3). It is of interest that several recent studies link STAT3 to NF-κB signaling (18, 36). In the present study we show that in cells expressing the WT form of STAT3, such as Daudi cells and STAT3-reconstituted DRST3 cells, p50 is basally bound to the CXCL11 promoter, and upon IFN treatment p65 displaces p50 binding to the promoter. However, in STAT3-defective Daudi cells, IFN treatment induces the binding of p50 to the CXCL11 promoter, whereas p50 protein is not basally bound. This result is in contrast to other NF-κB-regulated ISGs heretofore examined in which p50 was found basally bound to the promoters (21). Protein p50 differs from other NF-κB proteins in that it does not have a transcriptional domain and functions as a transcriptional repressor. In contrast p65 is a transcriptional activator and appears to up-regulate the expression of ISGs. Our previous study shows that in a gene-dependent manner NF-κB positively or negatively regulates gene expression (21). Initial studies using S50-KO MEFs indicate that the p50 subunit of NF-κB appears to negatively regulate the induction of CXCL11 expression (our unpublished observation). In agreement with our studies, the transcriptional induction of a CXCL11 promoter reporter construct by IFN-β suggested a critical role of NF-κB, specifically the p65 protein (35).

Moreover, we demonstrate for the first time a contrasting role for IRF1 and IRF2 in the IFN-induced expression of CXCL11. Although in IFN-sensitive Daudi cells and in STAT3-reconstituted DRST3 cells IFN induces the binding of IRF1 to the CXCL11 promoter, IFN induces the binding of IRF2 to the CXCL11 promoter in IFN-resistant DRST3 cells. IRF1 and IRF2 are members of the IRF family of transcriptional regulators that exert distinct roles in biological processes such as pathogen responses, cytokine signaling, cell growth regulation, and hematopoietic development (37). IRF1 and IRF2 have antagonistic roles in gene regulation: they bind to the same DNA elements, but IRF1 acts as a stimulator of transcription whereas IRF2 acts as a repressor (38). Apparently, alterations of the IRF1 to IRF2 ratio can have significant consequences for cell growth; restrained cell growth depends on a balance between these two mutually antagonistic transcription factors (39). Our results are consistent with the hypothesis that upon IFN treatment IRF1 activates CXCL11 gene transcription by directly binding to the ISRE site, whereas IRF2 binding to the CXCL11 promoter represses its transcription.

In summary, we show that CXCL11 is a gene target of STAT3 during IFN signaling. The IFN-induced expression of CXCL11 involves STAT3 but not the canonical Y705 dimerization site. A number of other factors are involved in IFN-induced CXCL11 expression including the binding of NF-κB proteins (p50 and p65) and IRF proteins (IRF1 and IRF2). Moreover, p50 and p65, as well as IRF1 and IRF2, have antagonizing roles in CXCL11 expression.

Acknowledgments

We thank Lawrence Blatt and Darren Baker for generously providing recombinant human IFNα and IFN-β. We also thank Sandhya Rani and Richard Ransohoﬀ (Cleveland Clinic Foundation, Cleveland, OH) for useful discussions during initial studies on CXCL11 gene expression in Daudi and DRST3 cells.

Disclosures

The authors have no financial conflict of interest.

References


