STAT-Phosphorylation–Independent Induction of Interferon Regulatory Factor-9 by Interferon- β

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Type I interferon (IFN)-dependent STAT1 and STAT2 activation requires specific tyrosine residues (337Y and 512Y) located in the cytoplasmic domain of IFNAR-2c, the β -subunit of the human type I IFN receptor. To identify STAT activation-independent induction of ISGs, we used a mutant cell line in which both 337Y and 512Y were substituted with phenylalanine (337F512F or FF mutant). In these cells, type I IFN failed to activate STAT1, STAT2, and STAT3 did not induce well-characterized ISGs and did not exert antiviral or antiproliferative effects. Using Oligonucleotide array (AffymetrixTM) analysis, we showed that interferon regulatory factor-9 (*IRF-9*) was the only gene induced by IFN- β in FF cells. Transient transfection analysis using an *IRF-9* promoter–reporter luciferase construct in FF cells confirmed induction of the *IRF-9* transcription unit by IFN- β . EMSA analysis using an IFN-stimulated response element (ISRE)-like sequence on the *IRF-9* promoter detected 2 novel DNA-binding complexes induced in nuclear extracts of IFN- β -treated FF cells. Supershift experiments identified the proteins IRF-1 and C/EBP- β in the complex. These studies provide the first evidence that signaling pathways leading to gene transcription are activated by IFN- β independent of STAT phosphorylation.

Introduction

THE JAK-STAT PATHWAYS ARE NOW the major regulators of the transcription of the interferon (IFN)-stimulated genes (ISGs), whose protein products mediate the multiple biological responses to IFNs (Darnell and others 1994; Borden and others 2007). Type I IFN-dependent JAK-STAT signaling requires both the type I IFN receptor chains, IFNAR-1 and IFNAR-2c, and the 2 JAK kinases, JAK1 and TYK2 (Uze and others 1990; Novick and others 1994; Lutfalla and others 1995; Domanski and others 1998). IFN- α/β treatment activates the formation of trimetric transcription factor complex, ISGF3, comprised of STAT1, STAT2, and interferon regulatory factor-9 (IRF-9), which binds to the ISRE of many ISG promoters to stimulate their transcription (Darnell and others 1994; Stark and others 1998).

Binding of type I IFNs induces the aggregation of the receptor chains, leading to the phosphorylation of tyrosine (Y) residues located in the intracellular domain of each receptor chain. IFN-induced phosphorylation of the Y466 and Y481 on IFNAR-1 is required for the docking of STAT2 (Yan and others 1996). No human cells that lack IFNAR-1

exist. However, the role of IFNAR-2c in type I IFN signaling has been studied by expressing IFNAR-2c mutants in U5A cells (Russell-Harde and others 2000; Wagner and others 2002). These cells that express IFNAR-1 but lack IFNAR-2c fail to respond to type I IFN confirming the requirement of this receptor chain for IFN signaling (Lutfalla and others 1995). A mutant IFNAR-2c, with phenylalanines in place of the 7 tyrosines of cytoplasmic tail (7F mutant), failed to support type I IFN-dependent STAT activation, gene expression, antiproliferative and antiviral effects. However, JAK1 phosphorylation could still be detected in these cells (Russell-Harde and others 2000). In complementary studies, individual tyrosines were introduced into the 7F backbone. Surprisingly, presence of a single tyrosine at position either 337 or 512 was sufficient to restore a complete IFN response, equivalent to that observed in U5A cells rescued with expression of full-length IFNAR-2c (Wagner and others 2002).

The majority of type I IFN-induced ISGs requires only STAT proteins for their transcriptional induction. However, our recent work has focused on the identification and characterization of genes that require accessory signaling

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components in addition to the JAK-STAT signals in response to IFN- β (Rani and Ransohoff 2005). Using an IFNAR-2c mutant cell line (337F512F mutant, Fig. 1A), we report that the *IRF-9* gene is induced in response to IFN- β independently of STAT1, STAT2, and STAT3 phosphorylation, indicating the existence of a novel IFN-induced signaling pathway.

IRF-9 belongs to a family of structurally similar but genetically and functionally distinct DNA-binding proteins (Taniguchi and others 1995). IRF-1 and IRF-9 are activators of transcription, IRF-2 and IRF-8 are repressors, and IRF-3 and IRF-4 can both activate and repress transcription (Nguyen and others 1997). Gene-knockout studies have shown that IRF-9 plays an essential role in activation of ISGs and antiviral response (Holtschke and others 1996; Kimura and others 1996). As reported earlier, *IRF-9* is a component of the transcription factor, IFN-stimulated gene factor 3 (ISGF3), which binds to the IFN-stimulated response element (ISRE) located in the promoters of ISGs to induce gene transcription (Darnell and others 1994). This is the first report of a gene induced by IFN- β in the absence of STAT1, STAT2, or STAT3 phosphorylation. We also show a unique complex consisting of IRF-1 and CCAAT/enhancer-binding protein- β to be important for regulating *IRF-9* expression in response to IFN- β .

Materials and Methods

Cell culture and reagents

The 2fTGH fibrosarcoma cells, mutant U5A cells that lack the IFNAR-2c receptor, U5A cells transfected stably with

the wild-type IFNAR-2c (R2C), and the mutant IFNAR-2c (FF) cells in which tyrosines at 337 and 512 are substituted with phenylalanine were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Russell-Harde and others 2000). Purified recombinant IFN- β -1b (2.3×10^7 IU/mg) was from Berlex Biosciences (Richmond, CA). LY294002, SB203580, and PD98059 were obtained from Biomol Research Labs (Plymouth Meeting, PA). Purified recombinant IFN- γ was obtained from Genentech Inc. (South San Francisco, CA). Total and anti-phosphotyrosine STAT1, STAT2, and STAT3 antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Anti-IRF-1 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-C/EBP- β antibody and expression plasmid were described elsewhere (Roy and others 2002).

RNA isolation and RNase protection assay

Total RNA was prepared from IFN-treated cells using the TRIzol reagent (Invitrogen Inc., Carlsbad, CA). RNase protection assays were performed as described (Rani and others 1996). A 350 bp fragment of human *IRF-9* cDNA was amplified by PCR using the primers, hIRF-9F1—CGCTTTCACCTCCCCA GGTCCAGGCCCGCCC and hIRF-9R1—CATCCTGAGGCAGGAGTCAGC CCCAGCCT, and cloned into the pGEM-T vector (Promega Inc., Madison, MI) to generate the hIRF-9 plasmid. DNA was linearized with restriction enzyme *Bam*HI and transcribed *in vitro* using T7 RNA polymerase to generate a 400 bp antisense

FIG. 1. Phosphorylation of STAT1, STAT2, and STAT3 in the mutant IFNAR-2c (FF) and wild-type IFNAR-2c (R2C) cells. **(A)** A schematic representation of the tyrosine residues in the cytoplasmic domain of IFNAR-2c expressed in U5A cells. U5A cells expressing the wild-type IFNAR-2c with all 7 intact cytoplasmic tyrosine residues at 269, 306, 314, 318, 337, 411, and 512 (R2C); mutant IFNAR-2c in which all the above 7 tyrosines have been replaced with phenylalanine (7F); mutant IFNAR-2C with a single tyrosine at 337 (337Y); mutant IFNAR-2c with a single tyrosine at 512 (512Y); mutant IFNAR-2c with 2 tyrosines at 337 and 512 (337Y512Y) and mutant IFNAR-2c with 2 phenylalanines at 337 and 512 (337F512F) are shown. **(B)** FF and R2C cells were left untreated (—), or treated with interferon (IFN)- α (α) or IFN- β (β) (5,000 U/mL) or IFN- γ (γ) (1,000 U/mL) for 15 min and whole cell extracts prepared. Cell lysates were resolved by SDS-PAGE, blotted onto PVDF membranes, and subjected to immunoblotting using anti-phospho-STAT1, STAT2, or STAT3 (left set of 3 panels). Blots were stripped and re-probed with equivalent levels in both cell lines.

probe. Probes corresponding to ISG 6-16 and an internal control γ -actin were described elsewhere (Ackrill and others 1991; Muller and others 1993). The *IRF-9*, 6-16, and γ -actin probes protect 350, 190, and 130 nucleotide long transcripts, respectively.

Transient transfection and luciferase assays

A -1,050 bp fragment corresponding to the upstream regulatory region of murine *IRF-9* was amplified by PCR using genomic DNA as a template. The primers used for PCR were IRF-9F1—ATACGAGCTCCTT-GGTGGTCCTT and IRF-9R1—TTGGAAGATCTGATATATGAGAACTC with restriction sites *SacI* and *BglII* (underlined) for directional cloning of the DNA into the pGL3-basic vector (Promega Inc., Madison, MI) in which the expression of luciferase is controlled by the cloned *IRF-9* promoter elements. A shorter construct of -340 bp *IRF-9* DNA was also generated using the primers IRF-9F2-ATACGAGCTCGCTTGTGAATCTG and IRF-9R1 (sequence shown above). Transient transfection assays were done using the FuGENE reagent (Roche Applied Science, Nutley, NJ) according to manufacturer's instructions. In brief, 1 μ g of pGL3-IRF-9-1050 or pGL3-IRF-9-340 reporters were mixed along with 0.1 μ g of *Renilla* luciferase control vector and transfected into cells in 24-well plates in serum-free media for 6 h at 37°C followed by the addition of serum (final concentration 10%) (Rani and others 2007). After over night incubation, cells were fed with fresh complete medium and were treated with IFN- β for 16 h. Cellular lysates were monitored for the luciferase activity using a commercially available dual luciferase assay kit (Promega Inc., Madison, MI). The firefly luciferase activity was normalized to that of *Renilla* luciferase and expressed as fold induction over control (Rani and others 2007).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cells treated with IFN- β (5,000 U/mL) for various times as described earlier (Rani and others 2001). An oligonucleotide probe corresponding to the IFN- γ -activated transcriptional element (GATE) of *IRF-9* gene was used for these assays. The reaction products were resolved on 8% non-denaturing polyacrylamide gels, which were dried and analyzed by autoradiography. For supershift assays, the extracts were incubated first with the antibodies for 20 min followed by the addition of the radio-labeled probe.

Western blots

FF cells were treated with IFN- β (5,000 U/mL) for various times and whole cell extracts prepared as described earlier (Rani and others 2002). Proteins were separated by SDS-PAGE and adsorbed to PVDF membranes and probed with the indicated primary and secondary antibodies. Immunoreactive bands were visualized using the ECL reagents (Amersham Biosciences, UK). Tyrosyl phosphorylation was monitored by Western blot analysis with anti-phosphotyrosine antibodies.

Microarray analysis

The human 6800 GeneChip array containing unique 20-mer oligonucleotide probes to >6,800 genes (Affymetrix™,

Santa Clara, CA) was used for this analysis. FF cells were treated with recombinant IFN- β (1,000 U/mL) for 1, 3, or 6 h or reserved as controls. Total RNA was isolated and reverse-transcribed to cDNA using Superscript (Invitrogen Life Technologies, Carlsbad, CA), and subsequently converted to biotinylated cRNA using Enzo High Yield RNA Transcript labeling kit (Enzo Biochem, Farmingdale NY). After hybridization the gene chips were washed and stained with streptavidin-PE. Data analysis was performed using the Genespring 3.0 software (Agilent Technologies Inc., Santa Clara, CA). The data were normalized to the expression levels of *actin* and *GAPDH*. Genes induced ≥ 2 -fold were selected for further analyses with PCR.

Results

FF cells lack IFN- β -induced phosphorylation of STAT1, STAT2, and STAT3

Phosphorylation of tyrosine residues on IFNAR-1 and IFNAR-2c is essential for the activation of STAT1 and STAT2 in response to type I IFNs. U5A cells lack IFNAR-2c (Lutfalla and others 1995). A schematic of the various mutants of IFNAR-2c is shown in Figure 1A. U5A cells reconstituted with wild-type IFNAR-2c receptor (R2C) and its functionally inactive mutants lacking either all 7 tyrosines (7F) or tyrosines at 337 and 512 (Y³³⁷F/Y⁵¹²F or FF) were used for the impact on IFN- β inducible gene expression (Wagner and others 2002).

We first analyzed tyrosyl phosphorylation of STAT1, STAT2, and STAT3 in the FF mutant cells in response to IFN- α , IFN- β , or IFN- γ using Western blot analyses. None of the 3 STATs were phosphorylated in response to IFN- α or IFN- β , although all the STAT proteins were present in cells, suggesting a defective type I IFN signaling in these cells (Fig. 1B). However, STAT1 was phosphorylated in response to IFN- γ , demonstrating the intact type II IFN-induced signaling in FF cells (Fig. 1B). Cells expressing wild-type IFNAR-2c receptor (R2C) had an intact phosphorylation of STAT1, STAT2, and STAT3 in response to both type I IFNs (Fig. 1B). As predicted, STAT1 was phosphorylated only in response to IFN- γ in these cells (Fig. 1B). Unphosphorylated STAT1, STAT2, and STAT3 proteins were expressed at equivalent levels in both cell lines (Fig. 1B). The FF cells thus provided an ideal background for studying STAT phosphorylation-independent induction of ISG expression.

Gene expression by IFN- β in FF cells

In addition to JAK-STAT signaling, accessory signaling molecules can modulate the function of STATs or activate complementary signaling components (Rani and Ransohoff 2005). Induction of *CXCL11* and *TRAIL* mRNAs by IFN- β not only required signaling involving phosphorylation of JAK-STATs but also other accessory signaling molecules like NF- κ B and PI3K (Rani and Ransohoff 2005; Rani and others 2007). To identify genes induced in the absence of STAT phosphorylation by IFN- β , RNA was isolated from FF cells treated with and without IFN- β for 1 or 3 h. Oligonucleotide array analysis (Affymetrix™) identified a small subset of ISGs induced by IFN- β . However, real-time RT-PCR (results not shown) and RNase protection assays confirmed only *IRF-9* induction by IFN- β in FF cells (Fig. 2A). *IRF-9* was very weakly induced by IFN- β in 7F cells at 3 h, suggesting

FIG. 2. Induction of interferon regulatory factor-9 (*IRF-9*) in the mutant IFNAR-2c (FF) cells by interferon- β (IFN- β). (A) RNA was isolated from untreated cells (C) or cells treated with 1,000 IU/mL of recombinant IFN- β for 1 and 3 h, respectively. The following cell lines were examined: wild-type IFNAR-2c (R2C), FF, and 7F. RNase protection assay was performed using 20 μ g of RNA and analyzed using probes for *IRF-9* (protects 350 bp), *6-16* (protects 160 bp), and γ -actin (protects 110 bp). The figure shows an autoradiogram derived from one of three experiments. (B) Cells were treated with different doses of IFN- β for 6 h as indicated. RNase protection assay of total RNA (20 μ g) was performed using probes for *IRF-9*, *6-16*, and γ -actin. The figure shows an autoradiogram generated on a PhosphorImager, derived from one representative experiment (out of 3). (C) FF cells were treated with 1,000 IU/mL of IFN- β for periods of time indicated on the panel. The figure shows an autoradiogram derived from one representative experiment (out of 3).

a requirement for a tyrosine residue in the cytoplasmic domain of IFNAR-2c for IFN- β -mediated induction of *IRF-9* (Fig. 2A). A classical control ISG 6-16, whose expression required IFN-induced STAT1 and STAT2 phosphorylation and binding to the promoter was not induced in IFN- β -treated 7F or FF cells (Fig. 2A). In U5A cells expressing the full-length IFNAR-2c receptor (R2C) induction of *IRF-9* and *6-16* mRNA occurred in response to IFN- β (Fig. 2A). These results indicate that optimal induction of *IRF-9* by IFN- β requires phosphorylation of STAT1 and STAT2 plus accessory signaling. However, it is clear that accessory signaling components independent of STAT activation can mediate *IRF-9* gene induction by IFN- β in FF cells.

We next addressed the kinetics and dose dependence of induction of *IRF-9* by IFN- β (Fig. 2B). The induction of *IRF-9* by IFN- β treatment was dose-dependent between 20 and 500 U/mL (Fig. 2B). For kinetic studies, FF cells were treated with IFN- β (1,000 IU/mL) for various periods of time (Fig. 2C). Maximal induction of *IRF-9* mRNA was observed following a 6-h treatment with IFN- β , and *IRF-9* mRNA accumulation was unchanged thereafter over 12 h of IFN- β exposure (Fig. 2C).

Transcriptional induction of *IRF-9* by IFN- β in FF cells

To determine if induction of *IRF-9* occurred at a transcriptional level, a 1,050 bp fragment of the murine *IRF-9* gene upstream of the translation start site was cloned into the promoterless pGL3-basic vector (Promega Corp., Madison, WI) upstream of the firefly luciferase reporter gene to generate the plasmid *IRF-9-1050* (Weihua and others 1997a). A schematic of the promoter of mIRF-9 is shown in Figure 3A. The sequence of the mIRF-9 promoter revealed the existence of potential regulatory elements, which

included NF- κ B, GAS, AP-1, GATE, Myc/Max, and SP-1 elements. Transient transfection assays in FF cells demonstrated a 9-fold increase in *IRF-9* promoter luciferase activity after treatment with IFN- β (Fig. 3B). A reporter driven by the promoter of *ISG-561* gene (a type I IFN-induced gene), which requires phosphorylation of STAT1 and STAT2, was not induced in the FF cells (results not shown). However, in the R2C cells, there was equivalent induction of *IRF-9* promoter-reporter and *561* promoter-reporter luciferase activity by IFN- β (results not shown).

We previously identified NF- κ B as an accessory factor essential for the induction of *CXCL11* and *TRAIL* by IFN- β (Rani and others 2001). The *IRF-9* promoter contained a potential NF- κ B element. To further define the role of this element in the IFN- β response, we created a deletion-mutation construct comprised of a 340 bp region of the promoter, which included the NF- κ B, GAS, and SP-1 element (Fig. 3A). Transient transfection assays showed that the *IRF-9-340* promoter-reporter construct was not induced by IFN- β in FF cells, indicating that the IFN-responsive region of the *IRF-9* gene was located between -1050 and -340 (Fig. 3B). Further analyses revealed that this region contained an ISRE-like sequence.

Novel IFN- β -induced DNA-binding complexes in FF cells

The GATE element in the promoter of murine *IRF-9* is essential for its transcriptional induction by IFN- γ (Weihua and others 1997a). High concentrations of IFN- α/β were required for GATE-mediated *IRF-9* induction in a human monocytic cell line (Xiao and others 2001). The GATE element shares some homology with the classical ISRE element that binds the transcription factor ISGF3 (Xiao and others 2001). The nucleotide sequences of the wtGATE and ISRE consensus are shown in Figure 4A. Two conserved nucleotides in

FIG. 3. Induction of interferon regulatory factor-9 (*IRF-9*) by interferon- β (IFN- β) requires the upstream region between -1,050 bp and -340 bp. **(A)** A schematic of the *IRF-9* promoter with its putative regulatory motifs. Also shown are 2 schematics of *IRF-9* promoter-reporter luciferase constructs. **(B)** Mutant IFNAR-2c (FF) cells were transiently transfected overnight with a *mIRF-9-1050* or *mIRF-9-340* promoter-reporter luciferase construct together with *Renilla* luciferase control vector. The luciferase activity was determined and normalized to *Renilla* luciferase for transfection efficiency. The fold increase with IFN- β (filled bars) compared to untreated control (open bars) is shown for *IRF-9-1050* and *IRF-9-340*. Results represent an average of 3 experiments with triplicate samples for each treatment.

the ISRE element are absent in the GATE element suggesting that ISGF3 would not be bound to the GATE element (Fig. 4A). To determine whether GATE formed complexes with proteins in response to IFN- β , we performed an EMSA. Two IFN- β -inducible DNA-binding complexes were detected in these assays. We termed these factors BBF-1 (β -IFN-induced DNA-binding factor-1) and BBF-2 (β -IFN-induced DNA-binding factor-2) (Fig. 4B). Untreated FF or R2C cells showed very low levels of BBF-1 but not BBF-2 (Fig. 4B). Both complexes were more robustly induced by IFN- β in R2C than in FF cells (Fig. 4B). It is important to note that these complexes were detectably induced in FF cells. The 2 complexes were competed out by 50-fold molar excess of unlabeled wtGATE oligonucleotides but not by a mutated mGATE oligonucleotide (Fig. 4B), suggesting the induced DNA-protein complexes bound to the GATE element in a sequence-specific fashion.

To address if new protein synthesis were required for the formation of BBF-1 and BBF-2 complexes, the FF and R2C cells were treated for 30 min with the protein synthesis inhibitor cycloheximide (100 μ g/mL), prior to stimulation with IFN- β for 5 h. As shown in Figure 4C, the generation of both complexes required *de novo* protein synthesis in both FF and R2C cells. Also, basal expression of the complex BBF-1 was abrogated by cycloheximide treatment.

IRF-1 and C/EBP- β are components of BBF-1

To identify the factors that interact with GATE in response to IFN- β , antibodies against various members of IRF and STAT proteins involved in the IFN signal transduction pathway were used for supershifting the EMSA complexes. Nuclear extracts from R2C cells treated with IFN- β for 5 h were used in these assays, since they induced more abundant DNA-binding complexes than were observed in FF cells (Fig. 4B). A wide panel of antibodies against various STATs, p65, IRF-3, and c-myc had no effect on the mobility of BBF-1 and BBF-2 as shown in Figure 5A. Interestingly, anti-IRF-1 blocked the DNA binding to BBF-1 suggesting it was a component of the BBF-1 complex (Fig. 5A and 5B).

The CCAAT/enhancer-binding protein- β (C/EBP- β) is an important component of GATE-dependent gene transcription (Roy and others 2002). Anti-C/EBP- β partially blocked the formation of the BBF-1 complex and shifted the mobility of the BBF-2 complex in nuclear extracts obtained from IFN- β -treated cells (Fig. 5B).

Nuclear extracts from IFN- β -treated R2C cells also contained a high-molecular-weight band (Fig. 5A), which was completely supershifted with STAT2 antibodies (Fig. 5A). Since this complex was absent in FF cells, it was not studied further.

FIG. 4. Novel DNA-binding complexes induced in mutant IFNAR-2c (FF) cells by interferon- β (IFN- β). **(A)** The nucleotide sequence of the wild-type GATE element (wtGATE), mutated GATE element (mGATE, mutated residues are shown underlined), and the consensus sequence for an ISRE are shown. The nucleotide residues not conserved between ISRE and GATE are indicated by thick vertical lines and thin vertical lines indicate consensus. **(B)** EMSA analysis of nuclear extracts from FF and wild-type IFNAR-2c (R2C) cells treated with recombinant IFN- β (5,000 U/mL) for 2 or 5 h or reserved as untreated controls (–) were assayed. The 32 P-labeled GATE probe bound 2 complexes, BBF-1 and BBF-2. Results from one of three experiments are shown. Incubation with a 50-fold molar excess of unlabeled wild-type GATE oligonucleotide (wtGATE) competed out the complexes but not mutated GATE oligonucleotide (mGATE) prior to incubation with radiolabeled GATE probe. **(C)** IFN- β -induced DNA-binding complexes required *de novo* protein synthesis. FF and R2C cells were pretreated with cycloheximide (100 μ g/mL) for 30 min followed by treatment with or without IFN- β (5,000 U/mL) for 6 h or reserved as untreated controls.

To address if IRF-1 and C/EBP- β are capable of regulating *IRF-9* transcription in response to IFN- β , we co-expressed vectors coding for these factors into FF cells. Co-transfection of a C/EBP- β expression plasmid enhanced luciferase transcription from the *IRF-9* promoter-reporter in presence of IFN- β compared to a control transfected with an empty expression vector (Fig. 5C). These results further supported the involvement of C/EBP- β for IFN- β -mediated induction of IRF-9. In contrast, overexpression of IRF-1 expression plasmid suppressed induction of wtIRF-9 promoter-reporter luciferase activity by IFN- β (results not shown).

Discussion

The JAK-STAT pathways are the major signaling routes for IFN-induced transcriptional induction of ISGs (Stark and others 1998; Aaronson and Horvath 2002; Borden and others 2007). However, it has now become clear that the JAK-STAT pathway, which culminates in the formation of dimers of STAT proteins, each of which is phosphorylated on tyrosine, cannot by itself explain the full range of IFN-dependent biological effects in intact animals or different cell types (Ramana and others 2000a, 2000b; Pilz and others 2003; Wang and others 2003). Several lines of evidence support the concept that accessory signals generated by ligation of IFN receptors are biologically relevant and act

in cell-type-specific fashion: for example, mice lacking type I IFN receptors are more susceptible to viral challenge than mice lacking STAT1, implying STAT-independent signaling (O'Shea and others 2002). Therefore, only a limited portion of the biological complexity of the IFN system is captured by examining the essential JAK-STAT components. Examination of the accessory signals that modulate the functions of STATs or activate complementary signaling components will provide insight into the mechanisms of IFN-induced expression of ISGs (Rani and Ransohoff 2005).

Here we describe *IRF-9*, an ISG induced by IFN- β in the absence of STAT phosphorylation. STAT1, STAT2, and STAT3 were not phosphorylated by IFN- β in the FF cells (which express a mutant IFNAR-2c that lack tyrosine residue at 337 and 512). IFN-induced activation of STAT4, STAT5, or STAT6 was also absent in these cells (results not shown). We used deletion mutagenesis and transient transfection assays to show functional importance of GATE, a positive regulatory element, for regulating *IRF-9* expression. We demonstrated 2 sequence-specific GATE-binding complexes in nuclear extracts of FF cells treated with IFN- β . No STAT protein was part of this complex. Although GATE shows some homology to ISRE, proteins that bind to ISRE-like STAT1, STAT2, and IRF-9 did not bind to GATE. Supershift analysis revealed transcription factors IRF-1 and CAAT/enhancer-binding

FIG. 5. cEBP- β is a component of the interferon- β (IFN- β)-induced DNA complexes in mutant IFNAR-2c (FF) cells. **(A)** EMSA analysis was performed using nuclear extracts from wild-type IFNAR-2c (R2C) cells treated for 5 h with IFN- β (5,000 U/mL). Supershift assays were performed using anti-interferon regulatory factor (IRF)-3, 2 different antibodies to STAT1, anti-STAT2, anti-STAT3, anti-STAT5, anti-p65, anti-c-myc, and anti-IRF-1. **(B)** EMSA analysis was performed using the above extracts as explained in **(A)** and supershift experiments were done using anti-IRF-1 and anti-C/EBP- β . **(C)** Overexpression of cEBP- β enhanced IFN- β -induced transcription of *IRF-9*. FF cells were co-transfected with the wild-type *IRF-9* promoter-reporter luciferase construct (*wtIRF-9-1050*) alone or in combination with wild-type cEBP- β expression vector. Cells were stimulated with and without IFN- β (1,000 U/mL for 16 h). IFN- β stimulation status are indicated as "+" for stimulated cells and "-" for nonstimulation. The luciferase activity was determined and normalized to *Renilla* luciferase for transfection efficiency. The fold induction with IFN- β compared to untreated control is shown. Results represent an average of 3 experiments with triplicate samples for each treatment.

proteins (C/EBP- β) to be components of the GATE-binding complexes induced by IFN- β .

CAAT/enhancer-binding proteins (C/EBPs) belong to a family of leucine zipper transcription factors and are involved in cellular proliferation and differentiation in various tissues (Landschulz and others 1988; Poli 1998). The role of C/EBP- β in type I IFN-induced signal transduction pathways is unknown. However, C/EBP- β is essential for the induction of *IRF-9* by type II IFN, IFN- γ (Weihua and others 1997a,b; Roy and others 2002; Roy and others 2005). Overexpression of C/EBP- β in FF cells enhanced transcriptional induction of *IRF-9* luciferase by IFN- β . C/EBP- β binding to DNA requires phosphorylation on serine and threonine residues (Roy and others 2005).

The IFN-regulatory factors (IRFs) belong to a family of transcription factors with at least 10 members in humans and mice, each of which play distinct roles in biological processes as revealed by gene-targeting studies (Tamura and others 2008). They were initially characterized as transcriptional regulators of type I IFNs and IFN-inducible genes, which play a major role in adaptive and innate immune responses (Tamura and others 2008). The IRF-binding domain recognizes a DNA sequence corresponding to approximately IFN-stimulated response element (ISRE) half site (Darnell and others 1994). Our studies revealed that IRF-1 binds to the GATE element in the promoter of *IRF-9* in response to IFN- β . However, it is not clear how it is regulated.

Previous studies have demonstrated that IFNs can activate Ras-mitogen-activated protein kinase cascade in various cells (Berger and others 1997; Nishiya and others 1997; Sakatsume and others 1998). Our earlier work demonstrated a requirement of accessory signaling by PI3K and p38MAPK for the induction of ISGs-like β -R1 and *TRAIL* by IFN- β (Rani and others 2002; Rani and others 2007). Our studies using pharmacological inhibitors did not suggest a role

for PI3K, p38 MAPK, or ERK kinase in the transcriptional induction of *IRF-9* by IFN- β (results not shown).

Robust induction of *IRF-9* by IFN- β was seen in the parental 2fTGH cells or R2C cells, suggesting the maximal induction of *IRF-9* by IFN- β required the phosphorylation of the 2 STAT proteins STAT1 and STAT2. However, the induction of *IRF-9* by IFN- β in FF cells that lacked phosphorylation of STAT1, STAT2, and STAT3 was clearly dependent on accessory signaling by IRF-1 and C/EBP- β involving non-STAT pathways. Defining the mechanism of activation of C/EBP- β , an essential and newly identified component in IFN-signaling pathway can help further understand how accessory signaling drives gene induction in the absence of STAT phosphorylation. We propose these studies will help define how the outcomes of accessory signals are integrated with and without activated STATs and are important for mediating cell-type-dependent selective transcriptional responses.

Acknowledgments

This research is supported by grant PO1 CA062220 from the National Cancer Institute. We are especially grateful to George R. Stark for his support and many helpful discussions.

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Received 23 March 2009/Accepted 14 July 2009