A Novel c-Jun-dependent Signal Transduction Pathway Necessary for the Transcriptional Activation of Interferon-γ Response Genes

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The biological effects of interferon-γ (IFN-γ) are mediated by interferon-stimulated genes (ISGs), many of which are activated downstream of Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) signaling. Herein we have shown that IFN-γ rapidly activated AP-1 DNA binding that required c-Jun but was independent of JAK1 and STAT1. IFN-γ-induced c-Jun phosphorylation and AP-1 DNA binding required the MEK1/2 and ERK1/2 signaling pathways, whereas the JNK1/2 and p38 mitogen-activated protein kinase pathways were dispensable. The induction of several ISGs, including ifn-205 and iNOS, was impaired in IFN-γ-treated c-Jun−/− cells, but others, such as IP-10 and SOCS3, were unaffected, and chromatin immunoprecipitation demonstrated that c-Jun binds to the iNOS promoter following treatment with IFN-γ. Thus, IFN-γ induced JAK1- and STAT1-independent activation of the ERK mitogen-activated protein kinase pathway, phosphorylation of c-Jun, and activation of AP-1 DNA binding, which are important for the induction of a subset of ISGs. This represents a novel signal transduction pathway induced by IFN-γ that proceeds in parallel with conventional JAK/STAT signaling to activate ISGs.

Interferon-γ (IFN-γ) regulates a range of cellular activities including anti-viral and anti-microbial immunity, apoptosis, and cell cycle progression through the induction of IFN-stimulated genes (ISGs) (1). Typically, IFN-γ signaling proceeds through the ligation of surface receptors, sequential phosphorylation of JAKs and STAT1 on tyrosine 701 (Tyr-701), and translocation of STAT1 homodimers to the nucleus to bind IFN-γ-activated sequences (GASs) found in the promoter regions of many ISGs (1, 2). However, other signaling pathways are important for IFN-γ signaling. For example STAT1 requires phosphorylation on Ser-727 to achieve full transcriptional activity (3, 4). Additionally, microarray analysis revealed that approximately one-third of ISGs were still regulated by IFN-γ in the absence of functional STAT1 (5–7), and STAT1−/− mice were more resistant to virus infection than mice lacking expression of the IFN-γ and IFNα/β receptors (5, 8). Thus, IFN-γ can induce expression of ISGs by STAT1-independent mechanisms, and the activity of multiple intracellular pathways acting in parallel play an important role in the biological response to IFN-γ.

Aside from the JAKs, other kinases are activated in response to IFN-γ, including MAP kinases (9–11), phosphatidylinositol 3-kinase and AKT (12), Pyk2 (11), calcium/calmodulin-dependent protein kinase II (13), and protein kinase C isoforms (14, 15). However, these enzymes have predominantly been assessed for their ability to trigger phosphorylation of STAT1 on Ser-727, and little is known about their potential impact upon STAT1-independent gene transcription. IFN-γ can activate transcription factors other than STAT1, including, class II trans-activator (CIITA) (16–18), CCAAT enhancer-binding protein (CEBP)-β (19–21), and interferon-responsive factors (IRFs) (22, 23), but the induction of these factors occurs downstream of JAK-STAT1 signaling. There are conflicting reports regarding the involvement of the IKK/IκB/NF-κB pathway in STAT1-independent IFN-γ signaling (24–26). Recently, it was shown that activation of IKKα and IKKβ in the absence of NF-κB activity was important for the induction of a subset of ISGs following treatment with IFN-γ (26). However the signaling proteins and transcription factors downstream of IKK activation by IFN-γ were not identified, and although STAT1 was phosphorylated on Tyr-701 and Ser-727 in IFN-γ-treated IκBα

blast; IRF, interferon-responsive factor; EMSA, electrophoretic mobility shift assay; wt, wild type; ChIP, chromatin immunoprecipitation; IKK, IκB kinase. 
\(\beta\)-deficient cells, it was not determined whether this pathway activated the expression of ISGs in the absence of STAT1.

We previously found that IFN\(\gamma\) could stimulate AP-1 DNA binding activity (27) and herein investigated the molecular events leading to the activation of AP-1 following treatment with IFN\(\gamma\). IFN\(\gamma\) induced rapid phosphorylation of c-Jun and concomitant activation of AP-1 DNA binding activity that was independent of JAK1 and STAT1 but dependent on c-Jun expression. Activation of AP-1 did not require p38 MAP kinases or c-Jun N-terminal kinase (JNK) 1/2 but did require the MEK1/2-ERK1/2 pathway. We identified ISGs (\(i\)fi205, iNOS) that were not activated by IFN\(\gamma\) in c-Jun \(^-/-\) cells, suggesting that AP-1 DNA binding activity was an essential regulator of these genes. IFN\(\gamma\)-induced expression of i\(fi\)205 (but not iNOS) was suppressed in STAT1 \(^-/-\) cells; additionally, IFN\(\gamma\)-induced iNOS transcription was observed in JAK1-deficient MEFs. In contrast, the IFN\(\gamma\)-mediated induction of IP-10 and SOCS3 was suppressed in STAT1 \(^-/-\) cells but was unaffected by perturbation of signaling through MEK1/2, ERK1/2, and c-Jun. Chromatin immunoprecipitation (ChIP) assays revealed that IFN\(\gamma\) induced rapid binding of c-Jun to the iNOS promoter. Our data show for the first time that, in addition to activation of JAK/STAT1 signaling, IFN\(\gamma\) can mediate phosphorylation of c-Jun and activate AP-1 DNA binding through the ERK MAP kinase pathway independently of STAT1.

**MATERIALS AND METHODS**

**Cells and Reagents**—STAT1 \(^-/-\), JNK1 \(^-/-\)/JNK2 \(^-/-\) (JNK1/2 \(^-/-\)), c-Jun \(^-/-\), and matched immortalized wild type (wt) MEFs were described previously (28, 29). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 2 mM l-glutamine (JRH), and all tissue culture reagents were sterile and free of mycoplasma and pyrogens. Mouse recombinant IFN\(\gamma\) and complete protease inhibitors were purchased from Roche Diagnostics. Antibodies for the following targets were used: pErk1/2 (phosphorylated on Thr-202 and Tyr-204); p38 MAPK (phosphorylated on Thr-180 and Tyr-182); pSTAT3 (phosphorylated on Tyr-705); pJNK (phosphorylated on residues Thr-183 and Tyr-185) (Promega, Madison, WI); p38 MAPK, STAT3, c-Jun, JunD, c-Fos, ATF-2, JunB (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies for the following targets were used: pErk1/2, pJNK, pSTAT3, c-Jun, pJNK (phosphorylated on Thr-180 and Tyr-182); pSTAT3 (phosphorylated on Tyr-705); c-Jun; pc-Jun (phosphorylated on Ser-63) (Cell Signaling Technology, Beverly, MA). Primers to mouse SOCS3 (forward, 5’ CGCTTCCGCTCAAAGCGAG-3’; reverse, 5’-GCTTCTCCTGACCTGCG-3’), \(i\)fi205 (forward, 5’-ATATCCCCAGGTCTCATCTTGG-3’; reverse, 5’-GCTTCCATCTGACCGG-3’), IP-10 (forward, 5’-GCTTGGAAATCTCCCTGCAG-3’; reverse, 5’-GGCAATGATC- TCAACACCGTG-3’), iNOS (forward, 5’- ATATCCCAGGTCTCATCTTGG-3’; reverse, 5’-GCTTCTCCTGACCTGCG-3’), JNK1, JNK2, and STAT3 were designed using Primer Express 2 software (Applied Biosystems, Foster City, CA). Primers to mouse SOCS3 (forward, 5’ CGCTTCCGCTCAAAGCGAG-3’; reverse, 5’-GCTTCTCCTGACCTGCG-3’), \(i\)fi205 (forward, 5’-ATATCCCCAGGTCTCATCTTGG-3’; reverse, 5’-GCTTGGAAATCTCCCTGCAG-3’; reverse, 5’-GGCAATGATC- TCAACACCGTG-3’) and iNOS (30) were designed using Primer Express 2 software (Applied Biosystems, Foster City, CA). The ribosomal gene L32 (forward, 5’-TTTGGATTTGTTCC- ACAACGTCAG-3’; reverse, 5’-TGTGAGCGATCTCGG- CAC-3’) was used as the control gene.

**Chromatin Immunoprecipitation**—ChIP assays were performed essentially as described previously (31) using 5 \(\mu\)g of anti-c-Jun antibody or rabbit IgG control antibody (Santa Cruz Biotechnology). The abundance of specific sequences in ChIP

**FIGURE 1.** IFN\(\gamma\) induces AP-1 DNA binding in MEFs. EMSAs were performed using radiolabeled oligonucleotides containing AP-1 consensus sequence (A), a GAS consensus sequence (B), and nuclear extracts from wt MEFs treated with 100 IU/ml IFN\(\gamma\) for the indicated times. C, competition EMSAs were performed using excess unlabeled wild type (wt) oligonucleotides and mutant oligonucleotides containing mutations critical for AP-1 binding and nuclear lysates from wt MEFs treated with 100 IU/ml IFN\(\gamma\) for 30 min. AP-1 DNA binding activity was assessed by EMSA using nuclear extracts from MEFs treated with 1–1000 IU/ml IFN\(\gamma\) for 30 min (D) and early passage primary MEFs treated with 100 IU/ml IFN\(\gamma\) for 15–30 min (E). All data presented are representative of at least three independent experiments.
samples was quantitated using the SYBR Green® dye detection method (Applied Biosystems, Warrington, UK). Primers used for PCR reactions were: iNOS (forward, 5' CCC AGC CCA ATT ACT TGA TTT 3'; reverse, 5' CGT GTT TTG CCC TTG TCT GAG 3'). Primers used for PCR reactions were: iNOS (forward, 5' CCC AGC CCA ATT ACT TGA TTT 3'; reverse, 5' CGT GTT TTG CCC TTG TCT GAG 3') flanking the region (1150 to 1046) in the iNOS promoter, SOCS3 (forward, 5' GCT GAA TGG TCC TAC GTC CCT T 3'; reverse, 5' TAC AGT TCC AAG CAT CCC GTG 3') flanking the region (565 to 539) in the SOCS3 promoter were designed using Primer Express 2® software. Threshold cycle numbers (Ct) were measured in the exponential phase for all samples. Ct values were converted to relative values using the equation (10^(-ΔΔCt) = CΔt) for all ChIP samples. Final relative values for enrichment were calculated as percentage of input.

Ribonuclease Protection Assay—MEFs were stimulated as indicated, and RNA was isolated with TRIzol (Invitrogen) according to the manufacturer’s protocols. 5 μg of RNA was hybridized to the Jun/Fos ribonuclease protection assay probe set (BD Biosciences, San Diego, CA) and ribonuclease protection assays performed using a ribonuclease protection assay kit according to the manufacturer’s protocols (BD Biosciences). Results were visualized by autoradiography on x-ray film and quantitated on a phosphorimaging device using FX software (Bio-Rad). Statistical significance was tested using one-way analysis of variance testing with OriginLab version 7.5 software (Northampton, MA).

RESULTS

AP-1 Is Rapidly and Transiently Activated by IFN-γ—Using human tumor cell lines, we previously demonstrated that IFN-γ was capable of activating protein complexes that bound AP-1.
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We confirmed this in immortalized MEFs by EMSA using an oligonucleotide probe containing a consensus AP-1 site. After treatment of cells with IFNγ, we observed a rapid and transient increase in AP-1 DNA binding activity that returned to basal levels by ~120 min (Fig. 1A). The rapid induction of AP-1 DNA binding activity after IFNγ treatment was concomitant with the induction of GAS DNA binding activity (Fig. 1B). The IFNγ-induced AP-1 binding activity was specific, as it was competed by excess unlabeled oligonucleotides containing wild type (wt) (but not mutant) AP-1 binding sites (Fig. 1C). AP-1 activation by IFNγ was dose-dependent and could be induced by doses as low as 1 IU/ml (Fig. 1D). This effect is not confined to immortalized or transformed cells, as activation of AP-1 DNA binding activity by IFNγ was also seen in primary (early passage) MEFs (Fig. 1E).

IFNγ Activation of AP-1 Is Independent of JAK1 and STAT1—IFNγ-mediated activation of AP-1 DNA binding was independent of either JAK1 or STAT1 expression, because it could still be detected in JAK1−/− or STAT1−/− MEFs (Fig. 2). Interestingly, we observed that both basal and IFNγ-induced AP-1 DNA binding activities were enhanced in the STAT1- and JAK1-deficient cells. To determine whether other STAT complexes could compensate for the loss of STAT1 in our system, we studied GAS DNA binding activity in wt and STAT1−/− cells using the GAS element from the FcyRII gene (Fig. 2B). GAS binding species were detected in nuclear extracts from wt MEFs treated with IFNγ for 15–30 min but not in extracts from STAT1−/− cells, indicating they were dependent on the presence of STAT1. Similar data were obtained using the GAS element from the IRF-1 promoter (supplemental Fig. S1A) and the SIE from the c-fos promoter (data not shown). Furthermore, we detected no increase in expression or phosphorylation of STAT3 in IFNγ-treated STAT1−/− cells (supplemental Fig. S1B). We therefore propose that we have identified an IFNγ-induced, JAK1- and STAT1-independent signaling pathway leading to activation of AP-1 DNA binding.

c-Jun Is a Critical Component of the IFNγ-induced AP-1 Complex—AP-1 transcription factors are composed of Jun family (c-Jun, JunB, and JunD) homodimers, or Jun/Fos (c-Fos, FosB, Fra1, and Fra2) or Jun/ATF2 heterodimers. Ribonuclease protection assays showed that the Jun family genes and Fra1 and Fra2 were equivalently expressed in resting MEFs; however, almost no FosB or c-Fos mRNA expression was detected (supplemental Fig. S2, A and B). Moreover, none of the AP-1 subunit genes were induced by IFNγ (supplemental Fig. S2, C–E). Supershift assays with anti-
bodies specific for c-Jun, JunB, or JunD impaired the formation or migration of the IFNγ-induced AP-1/oligonucleotide complex, whereas antibodies against Fos family proteins or ATF2 had no effect (Fig. 3A). Expression of c-Jun was critical for the formation of the IFNγ-activated AP-1 DNA binding complex, as no AP-1 DNA binding activity was detected in c-Jun-/- MEFs treated with IFNγ (Fig. 3B). GAS binding activity was still evident in IFNγ-stimulated c-Jun-/- cells (data not shown), indicating that the JAK/STAT pathway remained intact. Optimal DNA binding activity by c-Jun requires phosphorylation of serine 63 (Ser-63) and serine 73 (Ser-73) (33). A minimal basal level of Ser-63-phosphorylated c-Jun was detected in untreated wt MEFs, which was robustly enhanced following exposure of cells to IFNγ (Fig. 3C). In contrast, we observed no consistent change in total c-Jun expression in response to IFNγ. Taken together, these data demonstrate that treatment of cells with IFNγ results in rapid phosphorylation of c-Jun and formation of an AP-1 DNA binding complex that contains c-Jun as an essential component.

The Activation of AP-1 by IFNγ Is Independent of JNK and p38 MAP Kinase but Requires ERK—JNK is the primary kinase responsible for the stimulated phosphorylation of c-Jun at Ser-63 and -73 (34). IFNγ-induced AP-1 DNA binding activity was detected in JNK1/2 double knock-out MEFs with similar kinetics and magnitude of induction to that seen in wt MEFs (Fig. 4A). Treatment with a specific peptide inhibitor of JNK (35) did not affect IFNγ-induced activation of AP-1 (Fig. 4B); however, this inhibitor effectively suppressed JNK-dependent activation of AP-1 following stimulation with tumor necrosis factor α (supplemental Fig. S3, A and B). In contrast to tumor necrosis factor α, IFNγ did not stimulate the phosphorylation of JNK1 and JNK2 (supplemental Fig. S3C), providing further supporting evidence that these kinases do not play a role in the IFNγ-induced activation of AP-1. p38 MAPK plays an important role in type I IFN signaling (9, 36); however, its role in IFNγ signaling is unclear (37, 38). We saw no phosphorylation of p38 MAPK following treatment with IFNγ, and SB203580 (a pharmacological inhibitor of p38 MAPK) had no effect on IFNγ-induced activation of AP-1 DNA binding (supplemental Fig. S4).

A previous study identified ERK1/2 as the kinases responsible for c-Jun phosphorylation in response to phorbol-ester or epidermal growth factor stimulation (39). Therefore, we tested whether the MEK/ERK pathway could mediate IFNγ-induced phosphorylation of c-Jun and activation of AP-1 DNA binding activity using the MEK1/2 inhibitor PD98059. PD98059 almost completely ablated the activation of AP-1 DNA binding activity following IFNγ treatment (Fig. 5A), suggesting that the MEK1/2-ERK1/2 pathway lies upstream of AP-1 DNA binding. This was supported by Western blot showing that ERK1/2 phosphorylation was detected within 5 min of IFNγ treatment (Fig. 5B) that was completely blocked by PD98059 (Fig. 5C). Moreover, PD98059 completely suppressed IFNγ-induced phosphorylation of c-Jun (Fig. 5D). These and supporting data obtained using the chemically distinct MEK1/2 inhibitor UO126 (supplemental Fig. S5) indicate that IFNγ can activate the MEK1/2-ERK1/2 signaling pathway resulting in c-Jun phosphorylation and induction of AP-1 DNA binding.

AP-1 Is Critical for IFNγ-mediated Induction of ifi205 and iNOS—To delineate the functional significance of the novel STAT-independent, IFNγ-stimulated MEK1/2-ERK1/2-AP-1 pathway that we had identified, we sought to identify ISGs that were regulated through this pathway. We selected candidate genes on the basis of their responses to IFNγ treatment in the presence or absence of STAT1 or AP-1 (3, 6, 7, 40) and performed quantitative reverse transcription-PCR to determine changes in expression in response to IFNγ. We identified ifi205 and iNOS as genes significantly activated in IFNγ-treated wt cells but not in IFNγ-treated c-Jun-/- cells, suggesting that c-Jun is essential for their induction by IFNγ (Fig. 6, A and B). In contrast, induction of SOCS3 and IP-10 by IFNγ was equivalent in wt and c-Jun-/- cells (Fig. 6, C and D). These experiments identify ifi205 and iNOS (but not
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**FIGURE 7.** Role of c-Jun in IFNγ-mediated induction of *ifi205*, *iNOS*, *IP-10* and *SOCS3*. Wild type (white bars) or STAT1−/− (black bars) MEFs were treated with 100 IU/ml IFNγ for either 1 or 6 h. RNA was extracted, cDNA synthesized, and quantitative real-time PCR performed with primers specific for *iNOS* (A), *ifi205* (B), *IP-10* (C), or *SOCS3* (D). E, wild type (white bars) or JAK1−/− (black bars) MEFs were treated with 100 IU/ml IFNγ for either 1 or 6 h. RNA was extracted, cDNA synthesized, and quantitative real-time PCR performed with primers specific for *iNOS*. Fold induction represents the mean fold change in transcription (untreated (Unt) cells were arbitrarily called 1) of three independent experiments, and error bars are the S.D. from the mean in these experiments. Statistical significance of fold gene induction relative to untreated samples was determined using one-way analysis of variance testing. *, p < 0.05; **, p < 0.01.

SOCS3 or *IP-10*) as genes requiring AP-1 activity for induction following treatment with IFNγ.

**JAK1 and STAT1 Are Dispensable for IFNγ-mediated Induction of iNOS**—We next determined whether the IFNγ-stimulated, c-Jun-dependent induction of *ifi205* and *iNOS* occurred in the absence of the classical JAK1/STAT1 pathway. *iNOS* was maximally induced in wt MEFs following 6 h of exposure to IFNγ (Fig. 7A), and a corresponding increase in *iNOS* protein was observed by Western blot (data not shown). In STAT1−/− (Fig. 7A) or JAK1−/− (Fig. 7E) MEFs, IFNγ induced *iNOS* mRNA to the same levels seen in wt cells; however, maximal induction was observed following 1 h of exposure to IFNγ and decreased slightly by 6 h, indicating that both JAK1 and STAT1 are dispensable for the induction of *iNOS* by IFNγ. In contrast, *ifi205* was significantly induced in wt cells exposed to IFNγ for 1 or 6 h but not in STAT1−/− cells (Fig. 7B). Thus, unlike *iNOS*, IFNγ-mediated induction of *ifi205* requires both AP-1 and STAT1. The induction of *SOCS3* and *IP-10* seen in IFNγ-treated wt cells was significantly attenuated in STAT1−/− cells (Fig. 7, C and D).

**IFNγ Induces Binding of c-Jun to the iNOS Promoter in Vivo**—Our experiments indicated that the MEK/ERK pathway and c-Jun were critical for the expression for ISGs such as *iNOS*, and the kinetics of the response were consistent with this being a direct transcriptional response downstream of c-Jun activation. We therefore investigated whether IFNγ activated the binding of c-Jun to the *iNOS* promoter using chromatin immunoprecipitation. The 1.7-kb regulatory region of the murine *iNOS* promoter contains two consensus AP-1 binding sites (40, 41), and we therefore chose PCR primers flanking these sequences. We also studied the IFNγ-responsive region of the SOCS3 promoter as an example of a c-Jun-independent gene promoter. Chromatin immunoprecipitation assays were performed using wild type and c-Jun-deficient MEFs after a 30-min exposure to IFNγ. We observed a marked increase in c-Jun binding to the *iNOS* promoter using chromatin immunoprecipitation (Fig. 8A, lanes 3 and 4). No binding was detected using a control antibody in the immunoprecipitation reaction (Fig. 8A, lane 2) or in c-Jun-deficient lysates immunoprecipitated with the anti-c-Jun antibody (Fig. 8A, lanes 5 and 6), demonstrating specificity of the reaction. When quantified by quantitative real-time PCR, there was an 18-fold enrichment of c-Jun bound to the *iNOS* promoter following IFNγ treatment (Fig. 8C). Consistent with the data shown in Figs. 6 and 7, no increase in binding of c-Jun to the *SOCS3* promoter was observed following treatment with IFNγ (Fig. 8B). These experiments demonstrate that c-Jun binds to the promoter of the *iNOS* gene following IFNγ treatment.

**DISCUSSION**

Induction of ISGs through JAK/STAT1 plays an important role in mediating the biological effects of IFNγ (1). However, other signaling pathways functioning in parallel with JAK/STAT1...
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STAT1 are required for a complete IFNγ response (5–7), as IFNγ can still induce ISGs, mediate anti-viral responses, and regulate cell proliferation in the absence of STAT1 (5, 6, 8). We have demonstrated that, in addition to activating STAT1, IFNγ stimulates the phosphorylation and activation of ERK1/2 through MEK1/2, resulting in the downstream phosphorylation of c-Jun, activation of AP-1 DNA binding, and the induction of genes such as \( \text{iNOS} \) and \( \text{ifi205} \) (Fig. 9). Importantly, we used chromatin immunoprecipitation assays to demonstrate the binding of c-Jun to the \( \text{iNOS} \) promoter following treatment with IFNγ. This signaling pathway was engaged by IFNγ in a range of human and mouse tumor cell lines, immortalized and primary MEFs, and in JAK1\(^{-/-}\) or STAT1\(^{-/-}\) MEFs. This indicated that, although IFNγ-mediated activation of the pathway was independent of JAK1 and STAT1, it was also fully active in JAK1/STAT1-expressing cells and thus did not merely serve as a compensatory mechanism in the absence of either JAK1 or STAT1. Moreover, the stimulation of AP-1 DNA binding activity following IFNγ treatment occurred rapidly and independently of the transcriptional induction of any AP-1 subunit genes, which is consistent with this pathway being a primary response pathway. Although the IFNγ-stimulated activation of AP-1 is independent of the classical JAK1/STAT1 pathway (and possibly all STAT proteins), we consistently observed that the loss of these proteins augmented basal levels of AP-1 DNA binding. It is possible that this is due to the loss of a negative regulation of IFNγ signaling controlled by genes in a JAK1/STAT1-dependent manner; however, this needs to be determined experimentally.

We have identified three classes of ISGs that differentially utilize the JAK/STAT1 and MEK/ERK/c-Jun pathways in response to IFNγ. Induction of genes such as \( \text{IP-10} \) and SOCS3 by IFNγ was significantly attenuated in STAT1\(^{-/-}\) cells but was unaffected in c-Jun\(^{-/-}\) cells, demonstrating the importance of STAT1 for the activation of these ISGs. We recently found that \( \text{ifi204} \) had similar transcriptional requirements (data not shown). In contrast, \( \text{iNOS} \) was induced by IFNγ in wt, JAK1\(^{-/-}\), and STAT1\(^{-/-}\) cells but not in c-Jun\(^{-/-}\) cells. Previously, it has been demonstrated that STAT1, NF-κB, IRF-1, and IRF-8 can play important roles in the induction of \( \text{iNOS} \) by the combined treatment of IFNγ and lipopolysaccharide in macrophages (42). However, the utilization of different transcription factors in this response can be cell type-dependent, as IRF-1\(^{-/-}\) pancreatic islet cells, chondrocytes, and hepatocytes (but not macrophages) showed normal \( \text{iNOS} \) induction in response to
lipopolysaccharide/IFNγ (43, 44). Our data confirm observations by others (45, 46) that iNOS expression is enhanced following treatment with IFNγ alone, although we found induction of iNOS in MEFs occurred within 6 h of IFNγ treatment, whereas studies in macrophages did not detect iNOS expression until 24 h post-IFNγ treatment (45, 46). A third type of response was represented by the induction of iF205 by IFNγ that required both STAT1 and c-Jun (as did MIP-1β, data not shown). It is possible that STAT1 and AP-1 function cooperatively on the iF205 and MIP-1β promoters to transactivate these genes; indeed functional interactions between STATs and other transcription factors are known to occur. For example STAT3 and c-Jun cooperate to induce expression of α (2) macroglobulin (47). Moreover, although IFNγ induced expression of C/EBPβ in STAT1−/− MEFs (6), STAT1 was required for transactivation of C/EBPβ target genes but not DNA binding (48). Although our data indicate that at least three classes of ISGs exist, genome-wide expression profiling will be required to confirm this and may identify other groups of ISGs, such as those repressed by STAT1, AP-1, or both factors following IFNγ treatment.

Our data show that IFNγ-induced activation of AP-1 DNA binding was independent of both JAK1 and STAT1. Others have reported that hyperactivation of STAT3 by IFNγ can compensate for the loss of STAT1 and mediate transcription of ISGs (49). Although our data does not entirely exclude this possibility, it does present strong evidence that this is not the case in our experimental system. First, AP-1 activity and corresponding iNOS expression was not only detected in IFNγ-stimulated STAT1−/− cells but also JAK1−/− cells in which IFNγ is incapable of activating STAT3 or GAS binding (49, 50). Second, STAT1−/− MEFs consistently lacked all of the IFNγ-induced GAS binding complexes observed in wild type cells across a range of oligonucleotide probes derived from promoters of different ISGs. Third, the induction of IP-10, SOCS3, and iF204 was attenuated in STAT1−/− cells, suggesting that, at least for these genes, the absence of STAT1 could not be compensated. Finally, in contrast to a previous study (49), we saw no increase in protein levels or the magnitude and duration of phosphorylation of STAT3 on Tyr-705 in STAT1−/− cells.

We are yet to identify the molecular events that occur upstream of MEK1/2 following stimulation with IFNγ. Our data suggest that JAK 1 is dispensable; however, it does not rule out the possibility of compensation from other JAK kinases (especially JAK 2), which is a focus of continuing studies. IFNγ can also stimulate a large number of signal transduction proteins including Src family kinases c-Src (49) and Fyn (51), CrkL and its downstream small G protein Rap1 (52), phosphatidylinositol 3-kinase and its effector kinase AKT (12), protein kinase C δ (15), and Pyk2 (11). The role of these molecules in IFNγ signaling has mainly been studied in the context of Ser-727 phosphorylation on STAT1; however, all of these proteins are upstream of ERK1/2 in other systems using various cytokines to stimulate signal transduction and thus are also potential activators of the IFNγ-induced MEK/ERK/AP-1 pathway described herein.

It is becoming increasingly clear that a comprehensive response to IFNγ involves multiple signaling pathways that cooperate to stimulate transcription of ISGs (53). The IFNγ-induced MEK/ERK/c-Jun signaling pathway that we have deciphered provides important additional information regarding the molecular events that may underpin IFNγ biology. Although the JAK/STAT1 pathway plays a fundamental role in the biological response to IFNγ, it is evident that proteins, such as MAP kinases that can phosphorylate STAT1 on Ser-727, are also required for the full activation of STAT1 (7, 53). ERK1/2 and other IFNγ-induced serine/threonine kinases can phosphorylate substrates other than STAT1 (7, 53), such as c-Jun (this study), and these may prove to play an important and, so far, undescribed role in IFNγ-induced gene expression and biology.

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REFERENCES

1. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
2. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
3. Varinou, L., Ramsauer, K., Karaghiosoff, M., Kolbe, T., Pfeffer, K., Muller, M., and Decker, T. (2003) Immunity 19, 793–802
4. Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
5. Gil, M. P., Bohn, E., O’Guin, A. K., Ramana, C. V., Levine, B., Stark, G. R., Virgin, H. W., and Schreiber, R. D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6680–6685
6. Ramana, C. V., Gil, M. P., Han, Y., Ransohoff, R. M., Schreiber, R. D., and Stark, G. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6674–6679
7. Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002) Trends Immunol. 23, 96–101
8. Shresta, S., Sharar, K. L., Prigozhin, D. M., Snider, H. M., Beatty, P. T., and Harris, E. (2005) J. Immunol. 175, 3946–3954
9. Goh, K. C., Haque, S. J., and Williams, B. R. (1999) EMBO J. 18, 5601–5608
10. Nguyen, V. A., Chen, J., Hong, F., Ishac, E. J., and Gao, B. (2000) Biochem. J. 349, 427–434
11. Takaoka, A., Tanaka, N., Mitani, Y., Miyazaki, T., Fujii, H., Sato, M., Kovarik, P., Decker, T., Schlessinger, J., and Taniguchi, T. (1999) EMBO J. 18, 2480–2488
12. Nguyen, H., Ramana, C. V., Bayes, J., and Stark, G. R. (2001) J. Biol. Chem. 276, 33631–33638
13. Nair, J. S., DaFonseca, C. J., Tjernberg, A., Sun, W., Darnell, J. E., Jr., Chait, B. T., and Zhang, J. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5971–5976
14. Choudhury, G. G. (2004) J. Biol. Chem. 279, 27399–27409
15. Deb, D. K., Sassano, A., Lekmne, F., Majchrzak, B., Verma, A., Kambhamputti, P., Uddin, S., Rahman, A., Fish, E. N., and Platanias, L. C. (2003) J. Immunol. 171, 267–273
16. Croce, M., De Ambrosis, A., Corrias, M. V., Pistoia, V., Ochoino, M., Meazza, R., Giron-Michel, J., Azzarone, B., Accolla, R. S., and Ferroni, S. (2003) Oncogene 22, 7848–7857
17. Giroix, M., Schmidt, M., and Descoteaux, A. (2003) J. Immunol. 171, 4187–4194
18. Xu, Y., Wang, L., Buttice, G., Sengupta, P. K., and Smith, B. D. (2004) J. Biol. Chem. 279, 41319–41332
19. Roy, S. K., Hu, J., Meng, Q., Xia, Y., Shapiro, P. S., Reddy, S. P., Platanias, L. C., Lindner, D. J., Johnson, P. F., Pritchard, C., Pages, G., Pouyssegur, J., and Kalvakolanu, D. V. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7945–7950
20. Roy, S. K., Wachira, S. J., Wei, H., Hu, J., and Kalvakolanu, D. V. (2000) J. Biol. Chem. 275, 12626–12632
21. Xiao, W., Wang, L., Yang, X., Chen, T., Hodge, D., Johnson, P. F., and Farrar, W. (2001) J. Biol. Chem. 276, 23275–23281
22. Sato, M., Taniguchi, T., and Tanaka, N. (2001) Cytokine Growth Factor Rev. 12, 133–142
23. Taniguchi, T., and Takaoka, A. (2002) Curr. Opin. Immunol. 14, 111–116
c-Jun-dependent Activation of IFN-γ Response Genes

24. Deb, A., Haque, S. J., Mogensen, T., Silverman, R. H., and Williams, B. R. (2001) J. Immunol. 166, 6170–6180
25. Cheshire, J. L., and Baldwin, A. S., Jr. (1997) Mol. Cell. Biol. 17, 6746–6754
26. Sizemore, N., Agarwal, A., Das, K., Lerner, N., Sulak, M., Rani, S., Ransohoff, R., Shultz, D., and Stark, G. R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7994–7998
27. Clarke, C. J., Apostolidis, V., Hii, L. L., Gough, D. J., Trapani, J. A., and Johnstone, R. W. (2003) J. Cell. Biochem. 89, 80–93
28. Sizemore, N., Agarwal, A., Das, K., Lerner, N., Sulak, M., Rani, S., Ransohoff, R., Shultz, D., and Stark, G. R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7994–7998
29. Clarke, C. J., Apostolidis, V., Hii, L. L., Gough, D. J., Trapani, J. A., and Johnstone, R. W. (2003) J. Cell. Biochem. 89, 80–93
30. Mitrasinovic, O. M., Perez, G. V., Zhao, F., Lee, Y. L., Poon, C., and Murphy, G. M., Jr. (2001) J. Biol. Chem. 276, 30142–30149
31. Weinmann, A. S., Bartley, S. M., Zhang, T., Zhang, M. Q., and Farnham, P. J. (2001) Mol. Cell. Biol. 21, 6820–6832
32. Johnson, G. L., and Lapadat, R. (2002) Science 298, 1911–1912
33. Rahmsdorf, H. J. (1996) J. Mol. Med. 74, 725–747
34. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
35. Barr, R. K., Kendrick, T. S., and Bogoyevitch, M. A. (2002) J. Biol. Chem. 277, 10987–10997
36. Platanias, L. C. (2003) Pharmacol. Ther. 98, 129–142
37. Ramsauer, K., Sadzak, I., Porras, A., Pilz, A., Nebreda, A. R., Decker, T., and Kovarik, P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12859–12864
38. Sun, D., and Ding, A. (2006) Nat. Immunol. 7, 375–381
39. Morton, S., Davis, R. J., McLaren, A., and Cohen, P. (2003) EMBO J. 22, 3876–3886
40. Xie, Q. W., Whisnant, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784
41. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992) Science 256, 225–228
42. Kleinert, H., Pautz, A., Linker, K., and Schwarz, P. M. (2004) Eur. J. Pharmacol. 500, 255–266
43. Blair, L. A., Maggi, L. B., Jr., Scarim, A. L., and Corbett, J. A. (2002) J. Biol. Chem. 277, 359–365
44. Shiraishi, A., Dudler, J., and Lotz, M. (1997) J. Immunol. 159, 3549–3554
45. Jaramillo, M., Naccache, P. H., and Olivier, M. (2004) J. Immunol. 172, 5734–5742
46. Xiao, H., Zhu, C., Li, H., Chen, F., Mayer, L., Ozato, K., Unkeless, J. C., and Plevy, S. E. (2003) J. Biol. Chem. 278, 2271–2277
47. Zhang, X., Wrzeszczynska, M. H., Horvath, C. M., and Darnell, J. E. J. (1999) Mol. Cell. Biol. 19, 7138–7146
48. Hu, J., Roy, S. K., Shapiro, P. S., Rodig, S. R., Reddy, S. P., Platanias, L. C., Schreiber, R. D., and Kalvakolanu, D. V. (2001) J. Biol. Chem. 276, 287–297
49. Qing, Y., and Stark, G. R. (2004) J. Biol. Chem. 279, 41679–41685
50. Rodig, S. J., Meraz, M. A., White, J. M., Lampe, P. A., Riley, J. K., Arthur, C. D., King, K. L., Sheehan, K. C., Yin, L., Pennica, D., Johnson, E. M., Jr., and Schreiber, R. D. (1998) Cell 93, 373–383
51. Uddin, S., Sher, D. A., Alsayed, Y., Pons, S., Colamonici, O. R., Fish, E. N., White, M. F., and Platanias, L. C. (1997) Biochem. Biophys. Res. Commun. 235, 83–88
52. Alsayed, Y., Uddin, S., Ahmad, S., Majchrzak, B., Druker, B. J., Fish, E. N., and Platanias, L. C. (2000) J. Immunol. 164, 1800–1806
53. Platanias, L. C. (2005) Nat. Rev. Immunol. 5, 375–386