ERK1 and ERK2 Activate CCAAT/Enhancer-binding Protein-β-dependent Gene Transcription in Response to Interferon-γ*

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Interferons (IFNs) regulate the expression of a number of cellular genes by activating the JAK-STAT pathway. We have recently discovered that CCAAT/enhancer-binding protein-β (C/EBP-β) induces gene transcription through a novel IFN response element called the γ-IFN-activated transcriptional element (Roy, S. K., Wachira, S. J., Weihua, X., Hu, J., and Kalvakolanu, D. V. (2000) J. Biol. Chem. 275, 12626–12632). Here, we describe a new IFN-γ-stimulated pathway that operates C/EBP-β-regulated gene expression independent of JAK1. We show that ERKs are activated by IFN-γ to stimulate C/EBP-β-dependent response. Sustained ERK activation directly correlates with C/EBP-β-dependent gene expression in response to IFN-γ. Mutant MK1, its inhibitors, and mutant ERK suppressed IFN-γ-stimulated gene induction through the IFN-γ-activated transcriptional element. Ras and Raf activation was not required for this process. Furthermore, Raf-1 phosphorylation negatively correlated with its activity. Interestingly, C/EBP-β-induced gene expression required STAT1, but not JAK1. A C/EBP-β mutant lacking the ERK phosphorylation site failed to promote IFN-stimulated gene expression. Thus, our data link C/EBP-β to IFN-γ signaling through ERKs.

Interferons (IFNs)1 regulate the antiviral, antitumor, and immune responses in vertebrates by inducing the transcription of a number of IFN-stimulated genes (ISGs). Induction of ISGs occurs primarily due to the activation of the JAK-STAT pathway (1, 2). Type I (IFN-α/β) and type II (IFN-γ) IFNs bind to distinct cell-surface receptors and activate the signals that up-regulate the expression of ISGs (2). Upon binding to their receptor, IFN-α/β induce the tyrosine phosphorylation of the cytoplasmic tails of receptor using the JAKs Tyk2 and JAK1. JAKs undergo tyrosyl phosphorylation prior to inducing the receptor phosphorylation. Activated JAKs phosphorylate the STAT2 and STAT1 proteins at critical tyrosines. The STAT2-STAT1 dimer dissociates from the receptor and forms a heteromeric complex with a DNA-binding protein, p48 or IFN regulatory factor-9 or ISGF3γ (3). This complex, known as ISGF3, binds to the IFN-stimulated response element of the ISGs and induces gene expression (1, 2). Ligand-activated IFN-γ receptor recruits the Janus kinases JAK1 and JAK2, which selectively stimulate the phosphorylation of STAT1 (4). STAT1 dimers then rapidly migrate to the nucleus and induce the expression of ISGs that contain a γ-IFN-activated site (1, 2). STAT activation does not require new protein synthesis and is short-lived, lasting no longer than 30 min after ligand/receptor engagement. It does not persist over longer periods of time, despite the presence of IFN in the extracellular environment (1, 2). Such deregulation may occur due to the action of tyrosine phosphatases (5, 6) or degradation of STATs by proteasome (7). The SOCS-1 (suppressor of cytokine signaling-1) protein has been shown to be critical for inhibiting IFN-γ responses in vivo (8).

Induction of ISGs by IFN-γ is far more complex than that of IFN-α/β, largely due to the facts that the temporal control of these genes is variable and that, in some cases, blockade of protein synthesis prevents their expression (9). These data suggest that IFN-γ may activate different transactivating factors in a JAK-STAT-dependent or -independent manner. Evidence for these pathways is only beginning to accumulate. For example, the repression of the c-myc gene by IFN-γ occurs via both STAT1-dependent and -independent mechanisms (10). A number of transcription factors such as IFN regulatory factor-1 (11), IFN consensus sequence binding protein (12), class II transactivator (13, 14), and RXF (15) activate specific sets of ISGs in an IFN-γ-dependent manner.

IFN-γ augments IFN-α/β-induced gene expression by up-regulating the gene encoding the p48 subunit of ISGF3 (16, 17). A central role for p48 in IFN-regulated pathways is highlighted by several observations. Certain oncogenic viruses down-regulate p48 expression to evade the action of IFNs (18), and its activity is inhibited in some human tumor cell lines (19). IFN-
γ-induced expression of the p48 gene is rather slow (12–18 h) and is inhibited by the protein synthesis inhibitor cycloheximide (16, 17, 20), indicating the involvement of a novel regulatory element and its cognate factors. Promoter analysis revealed that the p48 gene promoter has no γ-IFN-activated site, but instead contains a unique IFN-γ response element termed GATE (20). Although GATE is partially homologous to the IFN-stimulated response element, factors that bind to the IFN-stimulated response element do not bind to GATE. The activity of GATE-binding factors is modulated by IFN-γ and is inhibited by cycloheximide and staurosporine (20). These observations suggest that IFN-γ regulates not only the synthesis of GATE-binding factors, but also their post-translational modifications. We have recently identified one of these as the transcription factor CCAAT/enhancer-binding protein-β (C/EBP-β) (21), a regulator of acute-phase responses and cell differentiation (22, 23). Although overexpression of C/EBP-β alone elevates basal gene expression, treatment with IFN-γ further augments gene expression through GATE (21). Our recent study demonstrated the first time a role for C/EBP-β in IFN-γ-regulated gene expression. Since the JAK-STAT pathway is rapidly activated by cycloheximide and staurosporine (20), these observations suggest that IFN-γ regulates not only the synthesis of GATE-binding factors, but also their post-translational modifications.

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C/EBP-β binds to the consensus sequence TTNNNGAAT (14, 15). The nucleotide sequence of GATE is as follows: 5′-CCCCAGGAGATTGAACTTGGG-3′. Six of the nine nucleotides in this region of GATE are homologous to the consensus C/EBP-β-binding site. Mutation of the AAACCT nucleotides of wild-type GATE resulted in a loss of C/EBP-β binding. In addition, a shorter GATE (GAGGAGATTGAACTCTT) that encompassed the C/EBP-β-binding site (20) or the C/EBP-β-binding site of GATE alone did not support gene induction by IFN-γ (data not shown). Furthermore, the majority (80%) of the IFN-γ-induced response was lost upon mutation of the C/EBP-β-binding site. Thus, a full-length GATE including the C/EBP-β-binding site is critical for IFN-γ induction. In addition, a C/EBP-β mutant lacking the transactivation domain blunted GATE-dependent gene expression (21). These data together indicate that C/EBP-β is an important component of GATE-dependent gene expression.

In this investigation, we show that IFN-γ stimulates the transcriptional activity of C/EBP-β through activation of ERK1 and ERK2 MAPKs, but independently of r-Raf and c-Ras. Pharmacological and gene-specific dominant-negative inhibitors of MKK1, ERK1, and ERK2 block gene expression. Surprisingly, STAT1, but not JAK1, is required to activate the C/EBP-β-dependent IFN-γ response. Together, our study identifies a new IFN-γ signaling pathway that couples the ERKs (MAPKs) to C/EBP-β-dependent gene regulation.
RESULTS

IFN-γ-induced Gene Expression through GATE Is Inhibited by Protein Kinase Inhibitors—To understand the role of protein kinases in IFN-γ-stimulated gene expression through GATE, we studied the influence of staurosporine, a protein kinase inhibitor. The RAW macrophage cell line was transfected with the P4 reporter gene, in which a 74-base pair element from the p48 promoter drives the expression of the luciferase gene. Cells were treated with IFN-γ in the presence or absence of staurosporine. Although IFN-γ alone strongly up-regulated GATE-dependent gene expression, it was strongly blocked by staurosporine (Fig. 1A). Since C/EBP-β binds to GATE and induces gene expression (21), we examined whether staurosporine also blocks C/EBP-β-regulated gene expression. As expected, C/EBP-β strongly induced luciferase gene expression upon cotransfection with the P4 reporter compared with the vector-transfected cells (compare the scales of Fig. 1, A and B). Treatment of cells with IFN-γ further enhanced C/EBP-induced expression. Staurosporine treatment not only inhibited IFN-γ-augmented gene expression, but also yielded lower luciferase activity than that obtained with C/EBP-β alone. These data suggest that C/EBP-β undergoes phosphorylation in response to IFN-γ treatment.

Since staurosporine is a general protein kinase inhibitor and recent reports indicated that MAPks are activated during IFN treatment (39, 40), we examined the effect of different inhibitors of the MAPK pathways on IFN-γ-induced gene expression through GATE. Three inhibitors, PD98059 (an MKK1-specific inhibitor), SB202474 (a negative control inhibitor for MKK1), and SB202190 (a p38 MAPK-specific inhibitor), were used in this study. RAW cells were transfected with the P4 reporter gene and then treated with a 25 μM concentration of each inhibitor prepared in Me2SO prior to IFN-γ treatment. PD98059, but not the negative control inhibitor SB202474, strongly blocked gene expression (Fig. 1C). SB202190 was marginally inhibitory at this dose, but this low-level inhibition is probably not specific since p38 MAPK is not activated under these conditions (see below). PD98059 inhibits activation of MKK1 and suppresses the phosphorylation of ERKs (41). We also studied the effect of a synthetic compound, U0126 (42), that specifically blocks the activated MKK1 function (i.e. ERK activation) on IFN-γ-induced gene expression. U0126 inhibited IFN-γ-induced gene expression in a concentration-dependent manner (Fig. 1D). Treatment with Me2SO (vehicle) had no effect on gene induction by IFN-γ. Thus, MKK1 activation appears to be necessary for IFN-γ-induced gene expression.

The effect of MKK1 inhibitors on endogenous p48 (ISGF3γ) gene expression was also studied. RAW cells were treated with IFN-γ in the presence or absence of either SB202190 or PD98059 for 16 h. Poly(A)+ RNA was extracted, Northern-blotted, and probed with p48 cDNA (Fig. 2A). IFN-γ strongly induced the expression of p48 mRNA (compare lanes 1 and 2). However, PD98059, but not SB202190, strongly inhibited IFN-γ-induced gene expression (compare lanes 3 and 4). A 6–8-fold reduction of p48 mRNA expression occurred. Neither inhibitor alone significantly altered p48 mRNA levels (lanes 5 and 6). Although the blot shows a marginal increase in the p48 mRNA signal with the inhibitors alone, this is not a reproducible effect. A reprobing of these blots with 32P-labeled β-actin cDNA revealed the presence of a comparable amount of RNA in all lanes (Fig. 2B). Thus, a reporter driven by p48 promoter elements and endogenous p48 behave in similar manner in the presence of the MKK inhibitor PD98059.

Inhibitors of MKK1 Block C/EBP-β-induced Gene Expression—Based on above results, we next examined the effect of MKK1 inhibitors on C/EBP-β-dependent gene expression. Cells were transfected with the P4 reporter and C/EBP-β expression vector and then treated with IFN-γ in the absence or presence of the indicated inhibitors. C/EBP-β itself induced gene expression, and IFN-γ, as expected, stimulated it very strongly (Fig. 1E). PD98059, but not SB202474, strongly inhibited C/EBP-β-dependent gene expression stimulated by IFN-γ. To ascertain the specificity of such inhibition, a similar experiment was performed using U0126. As expected, U0126 inhibited IFN-γ-stimulated expression in a concentration-dependent manner (Fig. 1F).

MKK1 Is Necessary for IFN-γ-induced Transcription through GATE—To directly demonstrate the role of MKK1 (MEK1), the effect of MKK1 coexpression on GATE-dependent gene expression was determined. Three different MKK1 expression vectors that carry the wild-type, constitutively active, or non-catalytic mutant MKK1 cDNA were employed in these experiments. A control transfection with the pCMV4 vector was also performed. Overexpression of the wild-type MKK1 cDNA alone slightly induced the luciferase gene compared with the vector control (Fig. 3A). Treatment with IFN-γ further stimulated the activity. The constitutively active MKK1 mutant elevated basal expression and augmented IFN-γ-induced expression to significantly higher levels compared with the wild-type MKK1 control. Thus, MKK1 may drive gene expression to some extent, using the basal levels of endogenous C/EBP-β. More importantly, the non-catalytic MKK1 mutant suppressed basal and IFN-γ-induced expression. These data indicate that MKK1 plays a critical role in IFN-γ-induced C/EBP-β-mediated signaling pathways.

MKK1 is downstream of Raf-1, a protein kinase (43, 44). In response to growth factors, Ras is first activated, followed by Raf. Therefore, we have studied the effects of dominant-negative Ras and Raf on GATE-driven gene expression (Fig. 3B). Cells were transfected with expression vectors carrying the constitutively active or dominant-negative mutants of Ras or c-Raf along with the P4 reporter. Coexpression of constitutively active Ras and Raf did not enhance IFN-stimulated luciferase gene expression compared with the vector controls. More importantly, neither dominant-negative Ras nor Raf inhibited gene expression. Instead, dominant-negative c-Raf slightly augmented IFN-γ-induced gene expression. To test the authenticity of the dominant-negative mutants, they were cotransfected with AP-1RE-Luc, in which the luciferase reporter gene is controlled by the consensus AP-1RE. As expected, EGF induced the expression of the luciferase reporter. However, in the presence of mutant Ras or Raf, EGF-induced luciferase expression was suppressed significantly (Fig. 3C). These mutants also blocked EGF-induced ERK phosphorylation in transient transfection assays.2 Taken together, these data indicate that Ras and Raf are not critical for IFN-γ-induced GATE-driven gene expression.

MKK1 Augments C/EBP-β-mediated Transcription—The effect of MKK1 on C/EBP-mediated transcriptional induction was examined next. RAW cells were transfected with the P4 reporter and a C/EBP-β expression vector. Along with these plasmids, wild-type, constitutively active, or non-catalytic mutant MKK1 was transfected, and luciferase activity was measured (Fig. 3D). C/EBP-β alone induced a low level of transcription, which was augmented by IFN-γ. Although IFN-γ further enhanced luciferase gene expression in the presence of wild-type or constitutively active MKK1, it failed to exert a similar effect in the presence of catalytically inactive MKK1. On the contrary, C/EBP-β-dependent gene expression was repressed in the presence of catalytically inactive MKK1. These data

2 P. S. Shapiro, unpublished observations.
indicate that MKK1 is critical for regulating C/EBP-β-dependent gene expression stimulated by IFN-γ.

The importance of MKK1 in IFN-γ signaling was further confirmed by use of U0126. Cells transfected with plasmids for P4-luciferase, constitutively active MKK1, and C/EBP-β were stimulated with IFN-γ in the presence or absence of U0126 (Fig. 3E). C/EBP-β-dependent gene expression was strongly induced in the presence of IFN-γ and constitutively active mutant MKK1, whereas U0126 completely ablated such gene induction. These data suggest that C/EBP-β is a downstream target of MKK1 signaling.

**ERK1 and ERK2 Are Activated by IFN-γ**—Since inhibition of MKK1 led to the suppression of IFN-γ-induced C/EBP-β-dependent gene expression, we next determined whether ERK1 (p44 MAPK) and ERK2 (p42 MAPK), the downstream substrates of MKK1 (43, 44), were also activated by IFN-γ (Fig. 4). In these experiments, IL-6 was used as a positive control because it is a known activator of C/EBP-β (45) and because it stimulates gene expression through GATE (46). RAW cells were treated with IFN-γ, and ERK phosphorylation was monitored on Western blots by probing an antibody that specifically detects the diphosphorylated (activated) form of ERK. Indeed, both ERK1 and ERK2 both were activated in response to IFN-γ (Fig. 4A). ERK1 activation by IFN-γ was slower (relative to IL-6) and occurred maximally only after 2 h of treatment, although slight activation was seen earlier. In contrast, IL-6 rapidly induced ERK phosphorylation within 30 min and dropped 2 h after treatment. IFN-γ-stimulated ERK1/2 activation persisted over longer periods of time (Fig. 4C) and could be observed up to 8 h. Such a pattern of ERK activation correlates well with induced C/EBP-β synthesis (Fig. 4G). As observed earlier (21), C/EBP-β protein was increased in cells after IFN-γ treatment. On these blots, a slower moving band that may represent the phosphorylated form of C/EBP-β could also be seen. These data are consistent with the slower induction of the p48 gene by IFN-γ.

ERK1/2 activation was also confirmed by immunoprecipitation with isoform-specific antibodies, followed by Western blotting with phospho-ERK-specific antibodies (data not shown). Finally, the functional activity of ERK was monitored in an in vitro kinase activity using myelin basic protein as substrate (Fig. 4F). Although a slight ERK activity was seen at 30 min and 1 h, maximal enzymatic activity was found after 2 h of IFN treatment. The differential kinetics of ERK activation suggests that IFN-γ employs a signaling cascade distinct from that of IL-6 and that the delayed ERK activation seems to be a ligand-
An IFN-γ-induced Signaling Pathway

Raf-1 Activity Is Not Necessary for IFN-γ-mediated ERK Activation—In the growth factor signaling pathways, Raf-1 activation precedes MKK1 phosphorylation (43, 44). Therefore, we examined whether MKK1 activation was dependent on Raf-1 in response to IFN-γ. Raf-1 was first immunoprecipitated from IFN-γ-stimulated cell extracts and then incubated with [γ-32P]ATP and bacterially produced MKK1. After the reaction proteins were separated by SDS-PAGE, they were transferred to a polyvinylidene difluoride membrane. Incorporation of radioactivity into MKK1 was monitored using a PhosphorImager. No significant Raf-1-induced phosphorylation of MKK1 occurred under these conditions (Fig. 5A), although a comparable amount of Raf was present in all the reactions (Fig. 5B). Quantification of the radioactivity in the MKK1 band showed a lower phosphorylation level compared with untreated cells (Fig. 5C).

We next examined whether the absence of Raf-1 phosphorylation in IFN-γ-treated cells was due to a lack of its phosphorylation. To demonstrate this more clearly, Raf-1 was immunoprecipitated from untreated and IFN-γ-treated cells, and the samples were separated by SDS-PAGE. Raf-1 from IFN-γ-treated cells (Fig. 5D) had a slower mobility compared with Raf-1 from untreated cells. Since Raf-1 phosphorylation may reflect its kinase function (43, 44), we monitored its activity in a coupled assay. Immunoprecipitated Raf-1 was incubated with [γ-32P]ATP, bacterially produced wild-type MKK1, and catalytically inactive ERK2 proteins. In this assay, ERK2 will be phosphorylated only if MKK1 is activated by Raf-1. Fig. 5E shows the radioactive phosphate incorporation into the substrates. No increase in MKK1 or ERK2 phosphorylation was observed when incubated with Raf-1 from IFN-γ-treated cells compared with Raf-1 from untreated control cells. Quantification of 32P in these bands revealed no significant difference in MKK1 and ERK2 phosphorylation between control and IFN-γ-treated cells (Fig. 5F, white bars). Thus, Raf-1 phosphorylation in response to IFN-γ does not correspond to an increase in its enzymatic activity. In contrast, EGF induced both Raf-1 phosphorylation (as seen by its slower mobility on the gel) and its kinase activity compared with the untreated cells (Fig. 5G). In these cells, EGF induced ERK1/2 phosphorylation as expected (Fig. 5H). The hypermobility of Raf-1 in response to IFN-γ and EGF appeared to be primarily due to phosphorylation since phosphatase treatment resulted in a loss of such mobility (data not shown).

ERK1 and ERK2 Mutants, but Not p38 Mutants, Block IFN-γ-induced Transcription—The functional relevance of ERKs in IFN-γ-induced transcription was examined next. RAW cells were transfected with the P4 reporter and an expression vector...
Fig. 4. ERK1 and ERK2 are activated by IFN-γ. RAW cells were treated with IFN-γ and IL-6 where indicated for defined lengths of time, and cell extracts were prepared as described under "Materials and Methods." In A, equal amounts of whole cell lysate from each sample were separated on SDS-polyacrylamide gels and Western-blotted. ERK activation was determined using phospho-ERK-specific antibodies. +Ve Control, 12-O-tetradecanoylphorbol-13-acetate-treated cell extracts. B shows the Western blot data obtained after stripping and reprobing of the blot shown in A with ERK2-specific antibodies. C shows the sustained ERK activation by IFN-γ over longer periods of time. Western blotting was performed as described for A. In D is shown a reprobing of the blot in C with antibodies specific for ERK1 and ERK2. Note a higher amount of ERK1 and ERK2 (2-fold) at 0 min (lane 1). E is a Western blot of immunoprecipitated ERK2. The positions of IgG and ERK2 are indicated. F shows the IFN-γ stimulation of the enzymatic activity of ERK2. ERK2 was immunoprecipitated from cell lysates after treatment with IFN-γ for the indicated times and incubated with MBP in the presence of [γ-32P]ATP. The reaction products were separated by SDS-PAGE and transferred to Western blots. 32P incorporation into the MBP band was quantified using a PhosphorImager. The fold increase in MBP phosphorylation relative to the 0-min control is shown below. G and H show the Western blots with C/EBP-β- and actin-specific antibodies, respectively. The numbers above the lanes indicate time (in minutes) after IFN-γ treatment.

Fig. 5. IFN-γ induces the phosphorylation of Raf-1, but not its kinase activity. Raf-1 was immunoprecipitated from IFN-γ-treated RAW cells (B) and incubated with bacterially produced MKK1 in the presence of [γ-32P]ATP for 30 min. MKK1 was visualized by autoradiography of the Western-blotted reaction components (A). Incorporation of 32P into MKK1 was quantified using a PhosphorImager (C). Time (in minutes) of IFN-γ treatment is shown. D–F, IFN-γ-induced hyperphosphorylation of Raf-1 does not activate kinase function. D shows immunoprecipitated Raf-1. The positions of Raf-1 and IgG are indicated on the right. Note the slow moving Raf-1 band in IFN-treated (120 min) cell extracts, but not in control cell extracts. Immunoprecipitated Raf-1 was incubated with bacterially produced wild-type MKK1 and kinase-dead ERK2 (ERK2-KD) in the presence of [γ-32P]ATP for 30 min. Following termination of reactions, the samples were separated by SDS-PAGE, Western-blotted, and autoradiographed. E shows the regions corresponding to MKK1 and kinase-dead ERK2. The quantitative representation of 32P in MKK1 (white bars) and kinase-dead ERK2 (black bars) bands is shown in F. C, untreated control; γ, IFN-γ (1000 units/ml). G–I, Raf-1 and ERK activation in EGF (40 ng/ml) for 30 min. In G, lysates prepared from untreated (U) and EGF-treated (E) cells were immunoprecipitated using Raf-1-specific antibodies as described for D. Numbers below the gel indicate -fold increases in Raf-1 kinase activity on its substrate MKK1. H and I are Western blots for ppERK1/2- and ERK1/2-specific antibodies in the same samples, respectively.

RAW cells were treated with IFN-γ and IL-6 where indicated for defined lengths of time, and cell extracts were prepared as described under "Materials and Methods." In A, equal amounts of whole cell lysate from each sample were separated on SDS-polyacrylamide gels and Western-blotted. ERK activation was determined using phospho-ERK-specific antibodies. +Ve Control, 12-O-tetradecanoylphorbol-13-acetate-treated cell extracts. B shows the Western blot data obtained after stripping and reprobing of the blot shown in A with ERK2-specific antibodies. C shows the sustained ERK activation by IFN-γ over longer periods of time. Western blotting was performed as described for A. In D is shown a reprobing of the blot in C with antibodies specific for ERK1 and ERK2. Note a higher amount of ERK1 and ERK2 (2-fold) at 0 min (lane 1). E is a Western blot of immunoprecipitated ERK2. The positions of IgG and ERK2 are indicated. F shows the IFN-γ stimulation of the enzymatic activity of ERK2. ERK2 was immunoprecipitated from cell lysates after treatment with IFN-γ for the indicated times and incubated with MBP in the presence of [γ-32P]ATP. The reaction products were separated by SDS-PAGE and transferred to Western blots. 32P incorporation into the MBP band was quantified using a PhosphorImager. The fold increase in MBP phosphorylation relative to the 0-min control is shown below. G and H show the Western blots with C/EBP-β- and actin-specific antibodies, respectively. The numbers above the lanes indicate time (in minutes) after IFN-γ treatment.

To rule out the role of other MAPKs in IFN-stimulated gene expression through GATE, gene expression was measured in the presence of a dominant-negative p38 kinase. Cells were transfected with the P4 reporter along with the pCMV5 vector or the same vector carrying a mutant cDNA of p38 MAPK. This mutant lacks the critical phosphorylation sites (TGY → AGF) and has been confirmed previously to exert a dominant-negative effect when overexpressed in the cells (40). IFN-γ-activated luciferase expression to a comparable level in the absence or presence of dominant-negative p38 (Fig. 6C). Thus, ERK blockade is sufficient for inhibiting IFN-γ-induced gene expression through GATE, whereas p38 kinase does not play a role in this process.

IFN-γ-stimulated C/EBP-β-dependent Gene Expression Re-
quires STAT1—Since STAT1 is essential for driving IFN-γ-induced responses (24), the role of STAT1 in IFN-γ-stimulated C/EBP-dependent transcription was determined using 2TGH (parental) and U3A (STAT1-deficient) cells (24). Cells were transfected with the P4 reporter in the presence or absence of C/EBP-β. C/EBP-β alone enhanced the transcription of the luciferase reporter compared with the vector (white bars) in 2TGH cells (Fig. 7A). Treatment of cells with IFN-γ strongly augmented transcription in C/EBP-transfected cells (black bars). Such augmentation of gene expression was above the levels observed in vector-transfected IFN-treated cells. A similar experiment conducted with U3A cells revealed two important pieces of information. 1) IFN-γ did not stimulate reporter gene expression; and 2) C/EBP-β-stimulated gene expression in the absence of STAT1 to a level comparable to that in the parental cell line, 2TGH. However, no hyperactivation of gene expression occurred in the presence of IFN-γ. Since U3A cells were derived by chemical mutagenesis, it is possible that mutations in genes other than STAT1 could cause a defective C/EBP-β response. Therefore, we have performed the same experiment with fibroblasts derived from STAT1 knockout mice (26). Whereas wild-type fibroblasts (WT SIM) showed enhanced C/EBP-β-dependent gene expression after IFN-γ treatment, STAT1−/− cells (SKIM) did not. However, basal transcription was elevated in the presence of C/EBP-β in these cells (Fig. 7B).

Based on these results, we next determined whether the stimulatory effect of IFN-γ on C/EBP-β-dependent gene expression could be rescued by transfecting an expression vector for STAT1 (Fig. 7C). The P4 reporter was cotransfected with C/EBP-β in the presence or absence of STAT1 in U3A cells. The cells were then treated with IFN-γ, and luciferase expression was determined. As expected, a basal level of C/EBP-β-dependent gene expression was observed in these cells, which was not stimulated further by IFN-γ. However, in the presence of STAT1, IFN-γ strongly induced C/EBP-dependent gene expression.

ERK1/2 Activation Is Dependent on STAT1—Since STAT1 and ERKs were both required for stimulating C/EBP-β-dependent gene transcription, we examined whether ERK activation occurred in STAT1-deficient cells after IFN-γ treatment. 2TGH cells were used as a positive control in these experiments. U3A cells were exposed to IFN-γ or IL-6 for various lengths of time, and ERK1/2 activation was monitored on Western blots using a phospho-ERK-specific antibody. Several differences were noted in the ERK activation profiles of 2TGH and U3A cells (Fig. 8A). In 2TGH cells, ERK1 and ERK2 were activated by 30 min by IL-6, but not by IFN-γ. At least 1 h of IFN-γ treatment was required for activating ERK1 and ERK2. However, in both cases, the activation was turned off by 2 h. Since U3A and 2TGH cells expressed similar levels of ERKs, these differences may not be due to different levels of ERK protein in the cells (Fig. 8B). In contrast, no ERK1 or ERK2 activation was noted in U3A cells in response to IFN-γ. However, IL-6 stimulated ERK in U3A cells (2–2.5-fold) compared with the untreated controls. Thus, ERK1 and ERK2 are activated in response to IFN-γ in other cell types with a characteristic lag compared with IL-6 treatment. However, the length
of the lag in IFN-γ-dependent ERK activation is cell type-depen-
dent. These data suggest that STAT1 is required for ERK
activation by IFN-γ, but not by IL-6. A slightly higher basal
ERK activity was seen in U3A cells. This may be due to fact
that these cells were derived after multiple rounds of chemical
mutagenesis and were grown in different medium from that
used for growing RAW cells. In addition, these cells grow faster
than RAW cells. The consistency of ERK activation was meas-
ured by Western blotting using phospho-ERK-specific antibodies. In A,
the same blot was stripped and then probed with ERK2-specific anti-
bodies. Each bar indicates the mean intensity in arbitrary units ± S.E. of triplicates. \(E\) shows the IFN-γ-stimulated ERK activa-
tion in wild-type (WTSIM) and STAT1−/− mouse fibroblasts. Note the
presence of a higher amount of ERK2 protein (middle panel) in lanes
4–6 (STAT1−/−) than in lanes 1–3 (WTSIM). The lower panel shows the
reprobing of the blot with anti-tubulin antibodies.

**FIG. 9.** JAK1-independent stimulation of C/EBP-β-dependent
gene expression by IFN-γ. In A and B, JAK1-deficient U4A (human)
or JKIM (mouse) fibroblasts were transfected with the P4 reporter
along with the C/EBP-β expression vector where indicated, and luci-
ferase expression was measured after IFN-γ treatment as in the legend
to Fig. 7. A parallel transfection was also performed in 2TGH (human)
and WTJIM (mouse) cells. C shows IFN-γ- or IL-6-induced ERK acti-
vation profiles in U4A cells. D shows the quantification of ppERK1
(white bars) and ppERK2 (black bars) in these cells. In E is shown the
effect of IFN-γ receptor mutations on ERK activation. The SCC mouse
fibroblast cell line expressing the wild-type or mutant human IFN-γ
receptor (28) was exposed to human IFN-γ (1000 units/ml) for the
indicated times. Cell extracts were Western-blotted using specific anti-
bodies directed against ppERK1 and ppERK2 (upper panel), ERK2
(middle panel), and tubulin (lower panel). E shows the expression of C/EBP-
b in the absence of STAT1. IFN-γ did not activate ERKs significantly in the
absence of STAT1.

**C/EBP-β-dependent gene expression**, we investigated whether
JAK1 was also critical for enhancing C/EBP-β-dependent gene
expression. To examine this aspect, U4A (JAK1-deficient) cells
(25) were transfected with the P4 reporter and C/EBP-β and
then treated with IFN-γ (Fig. 9A). As expected, C/EBP-β
strongly elevated basal gene expression compared with the vector
transfection. Treatment of C/EBP-β-transfected cells with IFN-γ
caused a strong up-regulation of luciferase expres-
sion, as in 2TGH cells. Similar results were obtained using
fibroblasts (27) derived from wild-type (WTJIM) and JAK1−/−
(JKIM) mice (Fig. 9B). Although the vector alone did not stim-
ulate C/EBP-β-dependent gene expression in JKIM cells, trans-
faction of C/EBP-β not only elevated basal expression, but also
enhanced gene expression in the presence of IFN-γ. This is in
contrast to the U3A and SKIM cells (Fig. 8), where the reporter
gene was not induced by IFN-γ in the absence of STAT1. These
data suggest that JAK1 is dispensable for the “activation” of
C/EBP-β by IFN-γ.

Based on the above results, we next determined whether
ERK1 and ERK2 were activated in U4A cells. Western blot
analysis was performed using a phospho-ERK-specific antibody. ERK1/2 activation was weaker (2-fold) in U4A cells and delayed (Fig. 9C) compared with 2TGH cells. The kinetics of ERK activation in U4A cells was similar to that in RAW cells. ERK enzymatic activity (MBP phosphorylation) also correlated with these data (data not shown). Interestingly, IL-6, which was active in the U3A cells, also caused a similarly delayed and weaker ERK activation. The increase in ERK activity was quantified in independent samples and is presented in Fig. 9D. The white and black bars show the ppERK1 and ppERK2 activation data, respectively.

The JAK1-independent activation of ERKs by IFN-γ was also determined in the SCC murine fibroblast cell line expressing the wild-type human IFN-γ receptor (hGR) or a mutant hGR that cannot bind JAK1 (hGR-LPKS → A). These cells express the ligand-binding α-chain and a portion of the human chromosome 21 that expresses the β-chain of the receptor (28). Cells were treated with human IFN-γ for various lengths of time, and ERK activation was measured (Fig. 9E). IFN-γ activated ERKs similarly in cells expressing the wild-type or mutant receptor. In these experiments, we noticed a higher activation of ERK2 over ERK1 due to the fact that these cells expressed lower amounts of ERK1. ERK activation by IFN-γ was slow (2 h), as in RAW cells. ERK1/2 activation by IFN-γ was quantified in independent samples (Fig. 9F).

**ERK Phosphorylation Site of C/EBP-β Is Critical for Stimulating Gene Expression in the Presence of IFN-γ**—To determine directly the role of ERKs in the phosphorylation of C/EBP-β, we employed two independent mutants of C/EBP lacking the ERK phosphorylation consensus sites (29, 30). In the first set of experiments, two mutants of murine C/EBP-β lacking the serine and threonine residues proximal to the basic leucine zipper domain were employed (Fig. 10A). Although these residues are at different positions (owing to different sizes of the protein) in the mouse and human C/EBP-β proteins, the sequence is highly conserved between the species. C/EBP-β Mut1, a mutant with alanines in place of serines distal to the amino terminus of the basic leucine zipper domain (29), responded normally to IFN-γ. However, Mut2, in which the ERK phosphorylation consensus sequence GTPS is converted to alanines, did not stimulate transcription upon IFN-γ treatment (Fig. 10B). A slight inhibition of basal transcription was noted in the presence of IFN-γ.

A similar result was obtained with a human C/EBP-β mutant (T235A) in which the threonine residue in GTPS was converted to alanine (30). Although wild-type C/EBP-β strongly induced reporter gene expression upon IFN-γ treatment, its corresponding mutant C/EBP-β lacking the critical threonine residue (T-A mutant) did not (Fig. 10C). These data suggest that ERK phosphorylation at threonine of C/EBP-β is essential for gene expression. This mutant inhibited basal gene expression.

To further prove that C/EBP-β is a downstream target of the MKK1-ERK pathway, RAW cells were transfected with wild-type or T235A mutant C/EBP-β along with constitutively active mutant MKK1. Cells were treated with IFN-γ, and luciferase activity was measured, as in Fig. 10B. A high basal and IFN-γ-induced expression was observed in cells transfected with wild-type C/EBP-β (Fig. 10D). However, mutant C/EBP-β failed to respond to IFN-γ. A low basal expression was noted in the cells transfected with mutant C/EBP-β, which was not induced further by IFN-γ. These data suggest that C/EBP-β is a downstream target of MKK1-activated ERKs.

**DISCUSSION**

Given the spectrum of IFN action, it is quite unlikely that only a specific set of genes regulate the antimicrobial, antitu-
In this study, we have identified an IFN-γ-regulated signaling pathway in which IFN-γ-activated ERK1 and ERK2 regulate C/EBP-β-dependent gene stimulatory effects. The temporal activation of ERKs correlated with the presence of C/EBP-β protein (Fig. 4G) and the previously described slower induction of the ISGF3γ gene (20). That activated ERK is necessary for IFN-γ-induced gene expression was confirmed using inactive ERK1 and ERK2 mutants. Although neither mutant alone blocked gene expression, together they strongly repressed IFN-γ-induced gene expression (Fig. 6). It is important to note that both ERKs were phosphorylated in response to IFN-γ (Fig. 4). Therefore, even if ERK1 is blocked by the corresponding mutant, ERK2 will still be active in cells transfected with kinase-inactive ERK1. The converse is true in cells transfected with the kinase-inactive ERK1 gene. The reason for such redundancy is unclear at this stage. It is likely that IFN-γ-activated ERK1 and ERK2 differentially control specific sets of genes.

The relevance of IFN-γ-dependent ERK activation to C/EBP-β was supported by several observations. 1) Wild-type human or murine C/EBP-β strongly induced gene expression through the p48 promoter in the presence of IFN-γ. 2) C/EBP-β mutants lacking the threonine residue in the ERK consensus sequence GTPS did not respond to IFN-γ (Fig. 10, B and C). 3) The M KK1 inhibitor U0126 blocked IFN-γ-stimulated C/EBP-β-dependent gene expression. 4) Constitutively active MKK1 augmented C/EBP-β-dependent IFN-γ-driven transcription (Fig. 3). However, it failed to activate a similar response in the presence of mutant C/EBP-β (Fig. 10D). This latter observation indicates that without a functional target, i.e., C/EBP-β, ERK signals cannot drive gene expression through GATE. Previously, a peptide derived from this region of C/EBP-β has been shown to serve as a substrate for ERKs (30). p38 kinase is not phosphorylated (data not shown), and a dominant-negative p38 kinase did not block gene expression induced by IFN-γ (Fig. 6).

These results indicate the specificity of M KK1-ERK for up-regulating gene expression through GATE. Interestingly, the stimulatory effect of constitutively active M KK1 on GATE-driven gene expression was further induced by IFN-γ. This result indicates that constitutively active M KK1 is still dependent on other IFN-γ-stimulated modifications or factors for inducing GATE-dependent gene expression. Furthermore, shorter GATE sequences (20) or those containing the minimal C/EBP-binding site of GATE alone (data not shown) did not permit the induction of luciferase by IFN-γ. The fact that a minor fraction (20%) of IFN-γ induction occurs in the GATE construct with a mutant C/EBP-binding site (data not shown) suggests that IFN-γ-stimulated ERKs may also modulate other factors, in addition to C/EBP-β.

ERK activation by IFN-γ differs from that by IL-6 (Fig. 4A). This is evidenced by the following. 1) STAT1 was required for IFN-γ (but not IL-6)-dependent ERK activation (Fig. 8, A, C, and D). 2) ERK activation was rapid in response to IL-6 in RAW and 2F1TCH cells, but slow in response to IFN-γ (Figs. 4A and 8A). 3) IL-6 did not activate ERKs over a longer period of time (Figs. 4A and 8A). That said, IL-6 and IFN-γ act similarly in other respects. IL-6 and IFN-γ caused a weaker but delayed ERK activation in JAK1-deficient cells (Fig. 9C). Slower activation of ERKs by IFN-γ also suggests that additional IFN-γ-controlled factors are necessary for ERK activation. Indeed, ERK activation by IFN-γ requires STAT1, but not JAK1. Thus, a STAT1-regulated factor is critical for C/EBP-β to sense the IFN-γ-induced transcriptional stimulus (Figs. 7 and 8). This factor could be a signaling enzyme or a coactivator of C/EBP-β-dependent gene expression. In an analogous manner, serine (but not tyrosine)-phosphorylated STAT1 has been shown to be critical for the steady-state expression of certain caspase genes (51). Consistent with this, we have isolated another factor that interacts with GATE. It is unlikely that STAT1 directly associates with C/EBP-β because electrophoretic mobility shift analysis did not reveal the presence of STAT1 in GATE-bound complexes (20). STAT1 did not co-immunoprecipitate with C/EBP-β in preliminary studies (data not shown).

Since IFN-γ activates ERKs slowly, this study cannot rule out a possibility that other intermediate factors may play a role in this process. For example, a recent study (52) has shown that the induction of cyclooxygenase-2 mRNA in normal human epidermal keratinocytes cells by IFN-γ in part involves transforming growth factor-α. In this case, both p38 MAPK and ERKs appear to be activated by transforming growth factor-α, as suggested by a chemical inhibition of these kinases and loss of cyclooxygenase-2 mRNA induction. In contrast, p38 MAPK is not required for C/EBP-dependent gene expression. These data suggest that induction of the p48 gene is different from that of the cyclooxygenase-2 gene. Future studies are required to analyze the involvement of any secondary factors. Our study used mutant fibroblasts and fibrosarcoma cell lines to define the mechanisms of control of C/EBP-β-dependent gene expression by IFN-γ. However, there could be some tissue-specific differences between fibroblasts and macrophages with respect to magnitude and duration of such responses. Since the p48 gene is induced by IFN-γ in most cell types, we believe that these data are generally applicable to different cell types.

We have noted a clear but weaker activation of ERK in JAK1−/− cells. Notably, ERK activation was delayed in JAK1−/− cells in response to IFN-γ and IL-6 compared with their wild-type counterpart (Fig. 9C). Thus, JAK1 may enhance the kinetics of ERK activation by IFN-γ, but is not critical for it. That JAK1 is not required for ERK activation is also supported by the data obtained with IFN-γ receptor mutants that cannot bind JAK1 (Fig. 9E). Similarly, in a previous study, kinase-inactive JAK1 was shown to sustain gene expression, but not the antiviral state (53). We have shown earlier that the endogenous p48 gene is not induced by IFN-γ in U4A and U3A cells (20). Consistent with this, another study has also shown that IFN-γ-induced p48 gene induction was suppressed in cells derived from STAT1−/− mice (54). Furthermore, the IFN-γ-induced expression of p48 and C/EBP-β genes was not detected in mouse fibroblast SKIM and JKIM cells, although it could be readily seen in wild-type cells (data not shown). This is because the GATE-binding factors are synthesized and/or modified in response to IFN-γ. Consistent with the properties of a GATE-binding factor, the expression and gene-inductive effects of C/EBP-β are induced by IFN-γ (21) and are dependent on JAK1 and STAT1 (data not shown). In the experiment shown in Fig. 9, C/EBP-β was constitutively expressed in JAK1−/− cells, thus “short-circuiting” its induction by IFN-γ. Once C/EBP-β protein is available, its functional activity becomes independent of JAK1, but dependent on IFN-γ. Thus, only one of the two steps involved in C/EBP-β-mediated gene expression is JAK1-dependent.

A previous study in HeLa cells has shown that Raf-1, but not Ras, is activated by IFN-γ (55). In contrast to that study, we found that Ras, Raf-1, and JAK1 are not required for IFN-γ-induced C/EBP-β-mediated gene expression through GATE. In contrast, the Ras and Raf mutants were able to repress EGFr-induced AP-1RE-driven transcription (Fig. 3C). This difference could be due to different cell types used in our study. However, unlike the previous study, we show here a functional target for the IFN-γ-activated ERKs. Although IFN-γ-dependent Raf-1

3 J. Hu, S. K. Roy, P. S. Shapiro, S. R. Rodig, S. P. M. Reddy, L. C. Platanias, R. D. Schreiber, and D. V. Kalvakolanu, unpublished data.
phosphorylation occurs in RAW cells, it appears to have an inhibitory effect on Ras-1 activity. Ras-1 did not activate its target MMK1 or the subsequent ERK phosphorylation (Fig. 5). Consistent with these results, dominant-negative Ras and Ras-1 subtly promoted GATE-dependent gene expression (Fig. 3B) instead of inhibiting it. In response to a number of extra-cellular stimuli, Ras-1 is phosphorylated at multiple serine and tyrosine residues (43, 44). Ras-1 phosphorylation does not always mean a positive regulation of its kinase function. A number of recent studies have shown that Ras phosphorylation negatively modulates its kinase function (56–60). In the proliferative signal transduction pathways, recruitment of activated Ras to the plasma membrane transiently stimulates its kinase activity (43, 44). Phosphorylation of Ras-1 decreases its affinity for the plasma membrane. Kinetically, Ras-1 phosphorylation correlates with a loss of function rather than a gain of function (57). IFN-γ seems to inhibit the proliferative signals by inducing Ras phosphorylation. The negative effect of IFN-γ on Ras-1 reflects its intrinsic nature. Ras-1 promotes cell growth and is an oncogene (59). In contrast, IFNs inhibit cell growth in a number of cell types (61). In agreement with these properties, we have observed a consistently lower Ras-1 kinase activity in IFN-γ-treated cells. In contrast, EGFR enhanced Ras-1 kinase activity (Fig. 5). C/EBP-β-like IFN-γ induces differentiation in a number of cells (23, 47). Since differentiation results in growth arrest, it is important to switch off the proliferative signals. This may be more critical for macrophages since their differentiation and action require C/EBP-β and IFN-γ (48, 49). All together, our study identified a new IFN-γ-regulated signaling pathway in which ERKs couple the signals emanating from the IFN-γ receptor to a versatile transcription factor.

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