The Med1 Subunit of Transcriptional Mediator Plays a Central Role in Regulating CCAAT/Enhancer-binding Protein-β-driven Transcription in Response to Interferon-γ

Hui Li†1, Padmaja Gade‡1, Shreeram C. Nallar‡1, Abhijit Raha†1, Sanjit K. Roy†1, Sreenivasu Karra†1, Janardan K. Reddy†1, Sekhar P. Reddy†1, and Dhananjaya V. Kalvakolanu†1,‡1

‡1 Department of Microbiology and Immunology, Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21205, the ‡Department of Pathology, Northwestern University School of Medicine, Chicago, Illinois, and the †Department of Environmental Health Sciences, The Johns Hopkins University School of Public Health, Baltimore, Maryland 21205

Transcription factor CCAAT/enhancer-binding protein (C/EBP)-β is crucial for regulating transcription of genes involved in a number of diverse cellular processes, including those involved in some cytokine-induced responses. However, the mechanisms that contribute to its diverse transcriptional activity are not yet fully understood. To gain an understanding of its mechanisms of action, we took a proteomic approach and identified cellular proteins that associate with C/EBP-β in an interferon (IFN)-γ-dependent manner. Transcriptional mediator (Mediator) is a multisubunit protein complex that regulates signal-induced cellular gene transcription from enhancer-bound transcription factor(s). Here, we report that the Med1 subunit of the Mediator as a C/EBP-β-interacting protein. Using gene knock-out cells and mutational and RNA interference approaches, we show that Med1 is critical for IFN-induced expression of certain genes. Med1 associates with C/EBP-β through a domain located between amino acids 125 and 155 of its N terminus. We also show that the MAPK, ERK1/2, and an ERK phosphorylation site within regulatory domain 2, more specifically the Thr189 residue, of C/EBP-β are essential for it to bind to Med1. Last, an ERK-regulated site in Med1 protein is also essential for up-regulating IFN-induced transcription although not critical for binding to C/EBP-β.

The CCAAT/enhancer-binding proteins (C/EBPs) are a group of structurally similar, but functionally and genetically distinct, transcription factors, which participate in a number of physiological activities. Their pleiotropic effects are regulated via various mechanisms, which include tissue- and embryonic developmental stage-specific expression, leaky ribosomal reading, post-translational modifications, and variable DNA binding specificities (1). Among its family, C/EBP-β exhibits a remarkable functional diversity and plasticity (1). In addition to the genes involved in energy metabolism, it also regulates interleukin-6 and interleukin-6-induced expression of the cytokines interleukin-1, interleukin-8, tumor necrosis factor-α, and granulocyte colony-stimulating factor as well as genes coding for α1-acid glycoprotein, α2-microglobulin, and C-reactive protein (2). A number of defects in cytokine synthesis were reported recently in cebpb−/− macrophages (3). Deletion of cebp in mice causes defects in macrophage-driven tumoricidal and bactericidal activities (4), T-helper 1 immune responses (5, 6), female fertility (7), glucose homeostasis (8), and the development and differentiation of hepatocytes (9), myelomonocytes (10), adipocytes (11), and neurons (12, 13). We have shown recently that C/EBP-β also plays an important role in regulation of IFN-induced gene transcription (14). Thus, it is unclear how a single transcription factor can drive such diverse processes. We hypothesized that dynamic association of cellular factors with C/EBP-β might ensure its activity in a gene context and signal-specific manner. Using IFN signaling as a model, we searched for cellular factors that associate with C/EBP-β using a proteomic approach. These studies identified Med1 (also known as TRAP220/PBP/DRIP220/CRSP220), a subunit of the transcriptional Mediator as an IFN-induced C/EBP-β-binding protein (15–17).

Here, we show that the N terminus of Med1 directly interacts with C/EBP-β in an IFN-inducible manner. Two serine residues, at positions 134 and 151, of Med1 appear to be critical for mediating these interactions and IFN-induced transcription. Additionally, inhibition of ERK1/2 or mutagenesis of an ERK phosphorylation site of C/EBP-β blocked its IFN-induced binding to Med1. Similarly, Med1 is also subject to regulation by ERKs. Mutations in the recently reported ERK1/2-regulated motifs of Med1 did not inhibit its interaction with C/EBP-β but suppressed IFN-induced transcription. These studies, thus, identify a novel regulatory aspect of C/EBP-β-driven transcription in response to IFN-γ.

MATERIALS AND METHODS
Reagents and Antibodies—Recombinant human and murine IFN-γ were purchased from PBL Biomedical Laboratories.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Mouse monoclonal antibody against FLAG tag and actin were obtained from Sigma. Rabbit polyclonal antibodies against C/EBP-β; goat polyclonal antibodies against Med1, Med23, Med24, and Med25; and bovine anti-goat IgG-horseradish peroxidase conjugate were purchased from Santa Cruz Biotechnology, Inc. Horseradish peroxidase conjugates of anti-rabbit and anti-mouse IgGs were obtained from GE Healthcare, Inc. ERK1-, ERK2-, and ppERK-specific antibodies (Cell Signaling Technology, Inc.) were used in this report. Rabbit polyclonal antibodies against the phospho-Thr189-C/EBP-β form of protein were provided by Peter Johnson (NCI-Frederick). The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. Protein was provided by Peter Johnson (NCI-Frederick). The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbiochem. The ERK pathway inhibitor U0126 (18) was purchased from Calbioch
mentioned otherwise, all primary antibodies were used at 1:1000 dilution, and secondary antibodies were used at 1:2000 dilution for Western blots. Signals were generated using ECL kits (Pierce). IP analyses were conducted as described earlier (31). Briefly, 350 μg of total cellular lysate was incubated with the desired antibody at 4 °C overnight and then incubated with protein G-agarose (Santa Cruz) at 4 °C for 2 h. Beads were washed, and bound proteins were resolved on 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) and checked by Western analysis.

**RESULTS**

**In Vitro Interaction Assay**—The pcDNA 3.1 vector carrying the mouse wild type cebpβ gene was employed as a template for generating an *in vitro* translated protein using a coupled TNT translation kit (Promega) and immunopurified using anti-C/EBP-β-specific IgG. This cell line was chosen because of its exquisite transcriptional specificity of C/EBP-β-participates in multiple transcriptional processes in response to disparate extracellular stimuli and plays a central role in IFN-γ-induced transcription in a number of cell types (14, 19, 33), we hypothesized that transcriptional specificity of C/EBP-β might be controlled by a stimulus-specific association with distinct cellular proteins. To identify the proteins that associated with C/EBP-β in response IFN-γ, we immunopurified total cellular proteins from IFN-stimulated RAW264.7 cells using C/EBP-β-specific IgG. This cell line was chosen because of its exquisite sensitivity to IFN (14). Since we were interested in obtaining a global picture of the IFN-stimulated C/EBP-β-binding proteins, we pooled the IP reaction products of IFN-stimulated samples from different batches. Proteins recovered from these IP reactions were trypsinized, and the resultant peptide mixture was subjected to MALDI-TOF analysis. Mass fingerprints of C/EBP-β-associated peptides from the IFN-stimulated cell extracts were used for querying the MASCOT protein fingerprint data base to predict the matches. Forty-three tryptic peptides from IFN-stimulated C/EBP-β-associated proteins matched to seven different proteins, and six nonoverlapping peptides (Table 1) from this mixture matched to that of Med1 protein. Mass fingerprints from unstimulated cells and/or isotypic IgG control did not reveal any Med1-derived peptides.

To further verify the specificity of these interactions and more importantly to examine if these interactions occur between the endogenous proteins in other cell types, isogenic cebpβ+/+ and cebpβ−/− MEFs were stimulated with IFN-γ, and cell lysates were subjected to IP with C/EBP-β-specific IgG. The IP products were probed for Med1 using an immunoblot. Both proteins interacted with each other in the steady-state in cebpβ+/+ cells, and IFN-γ treatment enhanced it by ~3-fold. Med1 protein was not detected in IP products of cebpβ−/− cells and in the IP products of control IgG. IFN treatment did not induce Med1 levels (Fig. 1A). Similarly, FLAG-tagged Med1 was able to associate with C/EBP-β like native Med1 in a variety of cells like HeLa, HEK-293, and hTERT-HME, indicating a cell type-independent interaction (Fig. S1). A genetic analysis of these interactions revealed that Med1 and C/EBP-β dynamically interacted with each other in the presence of IFN (Fig. S1).

Since Med1 is a part of a multiprotein complex, we next investigated if Med1 directly interacted with C/EBP-β. *In vitro* translated unlabeled C/EBP-β protein was incubated with an *in vitro* translated 35S-labeled Med1 protein. Protein translated from the Med1-programmed, but not mock, reactions bound to C/EBP-β (Fig. 1B). These interactions appear to be quite weak *in vitro*. Nonetheless, these data show that C/EBP-β can interact with Med1 in the absence of other constituents of the Mediator complex.

Med1 Is Required for IFN-induced C/EBP-β-dependent Expression of Certain Cellular Genes—Although the above experiments showed an IFN-induced augmentation of physical interactions between C/EBP-β and Med1, they did not reveal whether Med1 was required for IFN-induced expression of C/EBP-β-dependent genes. We have shown earlier that IFN-induced expression of irf9 mRNA is regulated by C/EBP-β (14). We have recently found that the death-associated protein kinase 1 gene (dapk1) is also regulated by IFN-γ through C/EBP-β (29). Therefore, we examined if IFN-γ-induced expression of these two genes was influenced by the loss of Med1 by stimulating isogenic med1+/+ and med1−/− cells with IFN-γ and monitored the expression levels of irf9 and dapk1 mRNA by real time PCR. Fig. 2A shows Western blot analysis of

**TABLE 1**

Med1-derived peptides identified by MALDI-TOF analysis

| Position | Sequence | Length |
|----------|----------|--------|
| 13       | MGSHPADNKTPS | 13     |
| 14       | MKNHQVATLIGS  | 14     |
| 16       | SKVQONPILSSLQIT | 16     |
| 11       | LLKDNPQDFS  | 11     |
| 15       | SKLPSTSDCFFQIT | 15     |
| 11       | TSTGSSPSQGS | 11     |

**RESULTS**

**Binding of C/EBP-β to Med1**—Since C/EBP-β participates in multiple transcriptional processes in response to disparate extracellular stimuli and plays a central role in IFN-γ-induced transcription in a number of cell types (14, 19, 33), we hypothesized that transcriptional specificity of C/EBP-β might be controlled by a stimulus-specific association with distinct cellular proteins. To identify the proteins that associated with C/EBP-β in response IFN-γ, we immunopurified total cellular proteins from IFN-stimulated RAW264.7 cells using C/EBP-β-specific IgG. This cell line was chosen because of its exquisite sensitivity to IFN (14). Since we were interested in obtaining a global picture of the IFN-stimulated C/EBP-β-binding proteins, we pooled the IP reaction products of IFN-stimulated samples from different batches. Proteins recovered from these
Mediator and C/EBP-β

[FIGURE 1. Med1 binds to C/EBP-β. A, interaction of endogenous Med1 with C/EBP-β in cebpβ+/+ and cebpβ−/− MEFs. Cells were stimulated with IFN-γ (500 units/ml) for 8 h, and then lysates were subjected to IP and WB analyses with the indicated antibodies. B, interaction of Med1 with C/EBP-β in the absence of other mediator subunits. In vitro translated purified C/EBP-β was incubated with mock-translated or Med1-programmed rabbit reticulocyte lysates as described under “Materials and Methods.” Med1 was translated in the presence of [35S]-labeled methionine and cysteine for detecting its expression. A fluorogram of the blot is shown in the top and middle of B.

[FIGURE 2. Med1 is required for the IFN-induced expression of the irf9 and dapk1 genes. Deletion of med1 or a knockdown of its expression resulted in a suppression of IFN-induced gene expression. The indicated cells were used for Western blot and real time PCR experiments. A and C, Western blot analyses with the indicated antibodies. B and D, real time PCR analyses for the indicated transcripts were performed (n = 9/sample). Data were normalized to the expression levels of rpl32. The mouse shRNAs do not target the endogenous human med1 mRNAs because of certain sequence differences. Thus, they served as a control in these experiments.

Med1 expression in med1+/+ and med1−/− MEFs. The IFN induced the expression of irf9 and dapk1 transcripts in med1+/+ cells (Fig. 2B) but not in med1−/− cells.

An shRNA-mediated knockdown of Med1 in wild type MEF (data not shown) and hTERT-HME1 cells (Fig. 2C) yielded similar data. Consistent with the knock-out cell data, IFN-induced up-regulation of irf9 and dapk1 mRNA levels was suppressed by med1-specific shRNAs and not by the controls (Fig. 2D). The steady-state expression level of these mRNAs was unaffected by med1-specific shRNA. Thus, Med1 is required for the IFN-induced expression of C/EBP-β-driven genes.

We next checked for Med1 recruitment to the dapk1 promoter in an IFN-stimulated manner, using ChIP assays. We used primers that could detect C/EBP-β binding to the critical IFN-induced regulatory element, CRE, of the dapk1 promoter (29). Since dapk1 was induced in a delayed manner by IFN-γ, med1+/+ and med1−/− MEFs were stimulated with IFN-γ for 8 h, and soluble chromatin was subject to the ChIP assay. IFN-induced recruitment of Med1 and C/EBP-β to the dapk1 promoter was seen in med1+/+ cells (Fig. 3A). No PCR product was detected in the controls, showing the specificity of the ChIP reaction. However, C/EBP-β was recruited to the promoter upon IFN treatment in med1−/− cells. This observation was further supported by a quantitative ChIP assay for Med1 recruitment to the dapk1 promoter upon IFN treatment (Fig. 3B). Last, restoration of f-med1, but not an empty vector, into med1−/− cells resulted in an IFN-induced recruitment of F-Med1 to the dapk1 promoter (Fig. 3B). When a similar experiment was performed in cebpβ+/+ and cebpβ−/− MEFs, Med1 was recruited to the dapk1 promoter following IFN-γ treatment only in the presence of C/EBP-β (Fig. 3A). This result was also confirmed by a quantitative ChIP assay (Fig. 3C). Consistent with this, when C/EBP-β was rescued into cebpβ−/− cells, Med1 recruitment to the dapk1 promoter was restored (Fig. 3C). Thus, IFN-induced recruitment of Med1 to the dapk1 promoter appears to be C/EBP-β-dependent.

Identification of the C/EBP-β-interacting Domain in Med1—Initial studies using the RBD-2 mutant, which failed to promote nuclear receptor-induced transcription, of Med1 indicated that NR-binding motifs are not essential for IFN-stimulated C/EBP-β-driven transcription and physical interaction (Fig. S2). Therefore, we next searched for the critical region that can bind to C/EBP-β by using several deletion mutants of Med1. The smallest of these constructs, C1, was able to associate with C/EBP-β. These studies lead to a conclusion that the first 155 amino acids of Med1 are critical for binding to C/EBP-β (Fig. S3).

Computer-based searches for conserved motifs in this region did not yield any clues. Therefore, we modeled the first 155 amino acids to obtain a conformation using Raptor protein folding software. The last 29 amino acids within this region have a propensity to fold into a small β-sheet and an α-helix (Fig. 4A). Based on these predictions, we substituted potential phosphoacceptor residues, such as Ser134 and Ser151 (bracketing the α-helical region), and Val125 and His127 residues (within the small β-sheet) to alanine in the context of C1 (Fig. 4B) and studied the impact of these sub-
Mediator and C/EBP-β

stitions on C1 interactions with C/EBP-β. Initial IP analyses were performed in HEK-293 cells. All three mutants expressed equivalently to that of C1 (Fig. 4C). However, all of them had significantly lost (about 80%) their ability to bind C/EBP-β compared with C1 (Fig. 4C). Unusually, the V125A/H127A double mutant ran slower than C1 in all experiments, under the conditions of electrophoresis. The reason for this anomalous migration was unclear, although there were no other sequence differences except for the mutant residues in this construct. In summary, disruptions within the last 29 amino acids of C1, specifically the Ser<sup>134</sup>, Ser<sup>151</sup>, and Val<sup>125</sup>/His<sup>127</sup> residues, severely affected C1 binding to C/EBP-β.

Effect of med1 Mutations on Transcriptional Induction of IFN-induced Genes Driven by C/EBP-β—Based on the information obtained from the C1 construct, we engineered the same substitutions into full-length Med1 and examined their impact on IFN-induced interactions with C/EBP-β vis-à-vis gene expression in med1<sup>−/−</sup> cells. Although all Med1 mutants expressed equivalently (Fig. 5A), their IFN-induced binding to C/EBP-β differed significantly from that of wild type Med1. No IFN-induced C/EBP-β binding was observed with S134A, S151A, and V125A/H127A mutants. However, the proteins coded by the S134D and S151D mutants bound to C/EBP-β like wild type Med1 upon IFN treatment (Fig. 5A). Similar results were obtained in HEK-293 cells (data not shown).

We next verified the functional significance of these interactions to IFN-induced C/EBP-β-dependent gene expression by measuring their impact on endogenous genes (irf9 and dapk1 mRNAs) and luciferase promoters driven by corresponding promoters. The mutant Med1 proteins significantly lost their ability to promote IFN-induced expression of dapk1 mRNA (Fig. 5B) and dapk1-luc (data not shown). Similarly, the recruitment of mutant Med1 proteins to dapk1 promoter was significantly inhibited (Fig. 5C). The S134A and H125A/V127A mutants also exhibited a lower steady-state activity compared with the wild type Med1, although this was not discernible in Western blots. Similar results were obtained with the irf9 gene (data not shown). No significant loss of activity was observed with S134D and S151D mutants albeit being lower compared with wild type Med1 upon IFN stimulation in all three methods analyzed.

To determine if the effect of Med1 mutations were specific to IFN-induced genes, we next measured their impact on DRS5-luc. All mutants induced luciferase activity upon RA treatment that was indistinguishable from that of wild type Med1 (Fig. 5D). We have also determined if defective association of Med1 with other Mediator subunits could account for its failure to activate transcription. Upon IP with FLAG tag-specific antibody followed by Western blot analysis, all Med1 mutants were able to associate with other members of the Mediator complex like wild type Med1 (Fig. 5E). Thus, Med1 mutations did not significantly affect its interactions with the other Mediator subunits.

The ERK1/2-regulated Site in Regulatory Domain 2 (RD2) of C/EBP-β Is Necessary for Its Interaction with Med1—We have previously shown that RD2 of C/EBP-β is necessary for IFN-induced gene expression (33). To further define the critical elements, we engineered mutations into RD2. The RD2 of C/EBP-β contains many serine and threonine residues, some of which are potential sites for phosphorylation (Fig. 6A). In the first set of experiments, we used two mutants, Mut1, which lacked the adjacent serine residues, and Mut2, which lacked the TPSP sequence. Both mutants were transfected into cebpβ<sup>−/−</sup> cells, and their IFN-induced binding to Med1 was compared with that of wild type C/EBP-β (Fig. 6B). Mut1 interacted with Med1 in a manner similar to that of wild type C/EBP-β following IFN treatment. However, Mut2 failed to bind Med1 in
response to IFN treatment. Such differential interaction was not due to differences in the expression levels of C/EBP-β or Med1 (Fig. 6B). In the second set of experiments, we used two other mutants of the GTPS motif, a consensus site for ERK1/2 phosphorylation. Mut T→A contained an alanine in place of threonine, and Mut T→D contained an aspartate in place of threonine. We have recently shown that this threonine residue is phosphorylated by ERK1/2 in response to IFN-γ treatment (29). Both mutants were transfected into cebpβ−/− cells, and their IFN-induced binding to Med1 was compared with that of wild type C/EBP-β (Fig. 6C). Unlike wild type, Mut T→A failed to bind Med1 above the steady-state level upon IFN-γ stimulation. In contrast, Mut T→D bound to Med1 readily in the steady state, and IFN treatment enhanced it further. Such differential interaction was not due to differences in the expression levels of C/EBP-β or Med1 (Fig. 6C).

A Role for ERK1/2 in Regulating IFN-induced Interplay between Med1 and C/EBP-β—To provide further evidence for ERK1/2 in regulating the interactions between Med1 and C/EBP-β, we initially studied the effect of U0126, a known inhibitor of ERK1/2 activation. This inhibitor not only blocked the IFN-induced phosphorylation of C/EBP-β at Thr189 but also inhibited the binding of Med1 to C/EBP-β (see Fig. S4).

This observation was further complemented by shRNA-mediated knockdown of ERK1/2 proteins. Wild type MEFs were infected with lentiviral particles containing shRNA that could target erk1/erk2 mRNAs. Greater than 95% of ERK1/2 was knocked down by the specific shRNA, not by the controls (Fig. 6D). These cells expressed comparable levels of C/EBP-β and Med1. Loss of ERK1/2 resulted in the IFN-induced interaction between C/EBP-β and Med1 mimicking the steady-state binding (Fig. 6E). Last, using ChIP assays, we measured the IFN-induced recruitment of C/EBP-β and Med1 to the dapk1 promoter. Both C/EBP-β and Med1 were recruited to the dapk1 promoter in an IFN-induced manner only in the controls but not in the presence of erk1/erk2-specific shRNA (Fig. 6F). Thus, ERK1/2 control the IFN-induced phosphorylation of Thr189 in the GTPS of C/EBP-β and subsequent recruitment of C/EBP-β and Med1 to the dapk1 promoter. Consistent with these results, expression of dapk1 mRNA was also inhibited in cells lacking erk1/erk2 (data not shown).

Like C/EBP-β, Med1 is also regulated by the MAPK pathways. A recent study showed that the Thr1032 and Thr1457 residues of Med1 are phosphorylated in response to thyroid hormone via an ERK-dependent pathway (34). Therefore, we next examined if these sites are also critical for mediating protein-protein interactions between Med1 and C/EBP-β. By mutating Thr1032 and Thr1457 residues to alanines. FLAG-tagged wild type and mutant Med1 constructs were transfected into med1−/− cells and were stimulated with IFN-γ. Lysates were subjected to IP with a C/EBP-β-specific IgG followed by a Western blot analysis with FLAG tag-specific antibody. Mutant proteins coded by the T1032A, T1457A, and T1032A/T1457A constructs bound to C/EBP-β like wild type Med1 (Fig. 7A). Thus, ERK1/2 phosphorylation sites of Med1 are not critical for its IFN-induced association with C/EBP-β. We next determined if these residues were required for supporting IFN-stimulated induction of dapk1-luc. All three mutants significantly lost their ability to promote IFN-induced luciferase expression from dapk1 promoter compared with wild type Med1 (Fig. 7B). The T1032A mutant had some residual transcriptional activity compared with T1457A. The T1457A mutant failed to activate IFN-induced transcription. These mutants also exhibited a similar regulatory profile in the context of dapk1 mRNA (Fig. 7C). These mutants yielded a similar picture, when tested in the context of irf9 promoter (data not shown). Thus, the Thr1032 and Thr1457 residues of Med1, although not impor-
The physiological diversity of C/EBP-β-driven responses suggests that multiple signal-induced post-translational modifications and the consequent interactions with cellular proteins may govern its cell-, gene-, and signal-specific effects. In order to understand these pathways, we first sought to identify cellular factors that associate with C/EBP-β in an IFN-γ-dependent manner. Our preliminary proteomic analyses have identified several proteins that participate in this process. In our strategy, we used pooled samples for detecting C/EBP-β-interacting proteins. One caveat with this approach is that low abundant proteins will not be detected because of pooling. Thus, it is likely that we have not detected all possible cellular proteins present in complex with C/EBP-β. On a similar note, not all proteins detected with this approach may be bound to C/EBP-β all of the time during IFN-γ stimulation. Nonetheless, our studies identified some significant IFN-induced C/EBP-β-interacting proteins.

The critical role of one such protein, Med1, in regulating IFN-induced transcription, has been demonstrated in this report using RNA interference, knock-out cells, IP, ChIP and mutational analyses. The Mediator protein complex regulates transcription from specific gene enhancers in response to hormones and other extracellular signals. Deletion of its constituent subunits leads to loss of a number of transcriptional events that participate in cell division, differentiation, and metabolism (21, 35–37). We have shown that Med1 dynamically and directly associates with C/EBP-β. The steady state binding of Med1 to C/EBP-β may regulate other IFN-independent C/EBP-β-regulated cellular genes. It is important to note that different transcription factors associate with distinct sub-
Mediator and C/EBP-β

units of the Mediator for modulating transcription in a signal-specific manner. One recent study showed that IFN-α-induced transcription driven by the STAT2 protein requires its interaction with the MED14 and MED17 subunits of Mediator (38). Thus, C/EBP-β does not appear to associate with the same subunits of Mediator complex, although it, like STAT2, functions in an IFN-regulated pathway.

Previous reports have shown that the N-terminal region of Med1 plays a critical role in mediating its interactions with other transcription factors, such as GATA1, GATA2, and Pit (21, 39). Although the exact contact sites for these transcription factors in the Med1 protein have not been finely mapped, a broad region of Med1 consisting of residues 622–701 appears to form a critical binding domain (39). This domain is distinct from the C/EBP-β binding region mapped in the current study. Last, the nuclear receptor binding LXXLL motif of Med1 is dispensable for binding to C/EBP-β (Fig. S2). We showed that the Ser134, Ser151, and Val125/His127 residues of Med1 are critical for promoting IFN-induced interaction with C/EBP-β (Fig. 5). These observations suggest that multiple residues of Med1 contact C/EBP-β for driving IFN-stimulated transcriptional response. Although the crystal structure of Med1 is unknown at this stage, the Ser134 and Ser151 residues flank a potential α-helix (Fig. 4). A negative charge at positions 134 and 151, probably acquired via phosphorylation, may allow it to interact with C/EBP-β efficiently. This interpretation is consistent with a loss of IFN-induced transcription upon conversion of these residues to alamines and a restoration of transcription following insertion of an aspartate residue. The two charged residues at Ser134 and Ser151 might serve as contact points, whereas the α-helix provides sufficient stretch for an interaction. These sites do not appear to be homologous to the consensus phosphorylation sites of the known protein kinases. Thus, it is unclear at this stage which kinase(s) might phosphorylate these sites. One of our future priorities is to identify the kinase that regulates phosphorylation at these sites. The Val125 and His127 residues are located in a predicted β-sheet-like structure that may form an additional interacting point of Med1. The equipotent activation of nuclear receptor-dependent transcription, but not IFN-induced transcription, by the Med1 mutants like the wild type protein suggests that functionally dissociable motifs mediate the interactions of Med1 with specific transcription factors.

We have also shown that the ERK1/2 signaling pathway is critical for promoting IFN-induced Med1 and C/EBP-β interactions and gene expression. Regulation of transcriptional coactivator proteins, such as CBP/p300, by MAPK kinase and other signaling pathways was shown earlier (40–42). A role for ERKs in regulating Mediator proteins was suggested earlier (43, 44). Recently, the Thr1032 and Thr1457 residues of Med1, direct substrates for ERK-dependent phosphorylation, have been shown to play an important role in regulating nuclear receptor-induced transcription (34). Although these sites are not critical for the IFN-induced binding of Med1 to C/EBP-β, they are necessary for driving IFN-induced transcription (Fig. 7). The C/EBP-β and nuclear receptor-induced transcriptional signals seem to functionally converge at these sites of Med1. Thus, we have mapped two separate domains of Med1, one that is required for binding to C/EBP-β and the other that mediates IFN-inducible transcriptional response. Like the Med1 protein, C/EBP-β also requires the MAPK signaling for promoting transcription (19, 33). A threonine residue, located within the RD2, of C/EBP-β plays an important role in mediating its interactions with Med1. We have shown that IFN-activated ERK1/2 can directly phosphorylate this residue (29). Thus, MAPK signaling controls phosphorylation of C/EBP-β and possibly of Med1. Both of these events are critical for ensuing transcription from IFN-responsive C/EBP-β-dependent gene promoters.

One recent study (45) has shown that C/EBP-β binds to the Med23 subunit of the Mediator. Our report identified Med1 as a C/EBP-β-interacting protein. Although the precise nature of this discrepancy is unclear, there are certain important differences between these two studies. The mouse C/EBP-β used in the current study is about 13 kDa smaller than its human counterpart and is significantly different from the chicken C/EBP-β used in those studies. Our studies used IFN-regulated promoters, whereas their studies investigated Ras-inducible C/EBP-β-dependent gene promoters. Another major contributing factor for these differences is the type of response element that is controlled by C/EBP-β. The irf9 gene is controlled by GATE, a unique response element, which is distinct from the conven-

![FIGURE 7. Effects of T1032A and T1457A substitutions on the interactions of Med1 with C/EBP-β. A, the indicated med1 mutants were subjected to IP and WB analyses after transfection into med1−/− cells and IFN-γ treatment as in other figures. B and C, effects of the indicated med1 mutants on the IFN-induced expression of dapk1-luc and endogenous dapk1 mRNA in med1−/− cells.](image-url)
tional C/EBP-β-binding sites (14, 28). In the case of dapk1, it is a CRE-like element that binds C/EBP-β in response to IFN-γ (29). These elements are distinct from consensus C/EBP-β-binding sites. In preliminary studies, we have also found that several non-Mediator proteins also form a complex with C/EBP-β in the presence of IFN-γ (data not shown). Whether these proteins influence the Mediator binding to different promoters or its composition is unclear. Unless these other C/EBP-β-interacting proteins are fully characterized, we may not know the precise reasons for these differences. Last, the studies of Mo et al. (45) did not rule out a role for additional Med proteins being part of a C/EBP-β-bound complex. Electron microscopy and other studies have shown that the Mediator complex is remarkably flexible with respect to its conformation and exists in different states (46–49), depending on the transcriptional activator. Furthermore, ligand-induced post-translational changes further contribute to these interactions. We have provided evidence for such activities in this report. The other possibility is that both Med1 and Med23 subunits of the fully assembled Mediator complex contact the C/EBP-β protein, which binds to DNA as a dimer. Such interaction may be driven IFN-induced transcription.

REFERENCES

1. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) J. Biol. Chem. 273, 28545–28548
2. Akira, S., and Kishimoto, T. (1997) Adv. Immunol. 65, 1–46
3. Gorgoni, B., Maritano, D., Martyn, P., Righi, M., and Poli, V. (2002) J. Immunol. 168, 4055–4062
4. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. (1995) Cell 80, 353–361
5. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Tattanazio, G., Biston, F., Frati, L., Cortese, R., Gulino, A., Ciliberto, A., Constantini, F., and Poli, V. (1995) EMBO J. 14, 1932–1941
6. Chen, X., Liu, W., Ambrosino, C., Ruocco, M. R., Poli, V., Romani, L., Quinto, I., Barbieri, S., Holmes, K. L., Venuta, S., and Scala, G. (1997) Blood 90, 156–164
7. Sterneck, E., Tesserollo, L., and Johnson, P. F. (1997) Genes Dev. 11, 2153–2162
8. Croniger, C., Trus, M., Lysek-Stupp, K., Cohen, H., Liu, Y., Darlington, G. J., Poli, V., Hanson, R. W., and Reshef, L. (1997) J. Biol. Chem. 272, 26306–26312
9. Diehl, A. M. (1998) J. Biol. Chem. 273, 30843–30846
10. Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Ishihiki, H., and Kishimoto, T. (1992) Blood 79, 460–466
11. Darlington, G. J., Ross, S. E., and MacDougall, O. A. (1998) J. Biol. Chem. 273, 30057–30060
12. Taubenfeld, S. M., Milekic, M. H., Monti, B., and Alberini, C. M. (2001) Nat. Neurosci. 4, 813–818
13. Menard, C., Hein, P., Paquin, A., Savelson, A., Yang, X. M., Lederfein, D., Barnabe-Heider, F., Mir, A. A., Sternek, E., Peterson, A. C., Johnson, P. F., Vinson, C., and Miller, F. D. (2002) Neuron 36, 597–610
14. Roy, S. K., Wachira, S. I., Weiwha, X., Hu, J., and Kalvakolanu, D. V. (2000) J. Biol. Chem. 275, 12626–12632
15. Myers, L. C., and Kornberg, R. D. (2000) Annu. Rev. Biochem. 69, 729–749
16. Malik, S., and Roeder, R. G. (2000) Trends Biochem. Sci. 25, 277–283
17. Rachez, C., and Freedman, L. P. (2001) Curr. Opin. Cell Biol. 13, 274–280
18. Favata, M. F., Horiuichi, K. Y., Manos, E. J., Daulerio, A. J., Bradlley, D. A., Feerer, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) J. Biol. Chem. 273, 18623–18632
19. Roy, S. K., Hu, J., Meng, Q., Xia, Y., Shapiro, P. S., Reddy, S. P., Platianis, L. C., Lindner, D. J., Johnson, P. F., Pritchard, C., Pages, G., Pouyssigur, J., and Kalvakolanu, D. V. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7945–7950
20. Jia, Y., Qi, C., Kashireddi, P., Surapureddi, S., Zhu, Y. J., Rao, M. S., Le Roith, D., Champon, P., Gonzalez, F. J., and Reddy, J. K. (2004) J. Biol. Chem. 279, 24937–24944
21. Crawford, S. E., Qi, C., Misra, P., Stellmach, V., Rao, M. S., Engel, J. D., Zhu, Y., and Reddy, J. K. (2002) J. Biol. Chem. 277, 35855–3592
22. Shui, J. W., and Tan, T. H. (2004) Genesis 39, 217–223
23. Ren, Y., Behre, E., Ren, Z., Zhang, J., Wang, Q., and Fendell, J. D. (2000) Mol. Cell. Biol. 20, 5433–5446
24. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. (1995) EMBO J. 14, 3170–3183
25. Minucci, S., Leid, M., Toyama, R., Saint-Jeannet, J. P., Peterson, V. J., Horn, V., Ishmael, J. E., Bhattacharyya, N., Dey, A., Dawid, I. B., and Ozato, K. (1997) Mol. Cell. Biol. 17, 644–655
26. Moffat, J., Grueneberg, D. A., Yang, X., Kim, S. Y., Kloepfer, A. M., Hinkle, G., Piqani, B., Eisenhaure, T. M., Luo, B., Grenier, J. K., Carpenter, A. E., Foo, S. Y., Stewart, S. A., Stockwell, B. R., Hacohen, N., Hahn, W. C., Lander, E. S., Sabatini, D. M., and Root, D. E. (2006) Cell 124, 1283–1298
27. Simpson, R. J. (2003) Proteins and Proteomics: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Weiwha, X., Kolla, V., and Kalvakolanu, D. V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 103–108
29. Gade, P., Roy, S. K., Li, H., Nallar, S. C., and Kalvakolanu, D. V. (2008) Mol. Cell. Biol. 28, 2528–2548
30. Kalakonda, S., Nallar, S. C., Lindner, D. J., Hu, J., Reddy, S. P., and Kalvakolanu, D. V. (2007) Cancer Res. 67, 6122–6120
31. Zhang, J., Yang, J., Roy, S. K., Tinninini, S., Hu, J., Bromberg, J. F., Poli, V., Stark, G. R., and Kalvakolanu, D. V. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9342–9347
32. Angell, J. E., Lindner, D. J., Shapiro, P. S., Hofmann, E. R., and Kalvakolanu, D. V. (2000) J. Biol. Chem. 275, 33416–33426
33. Hu, J., Roy, S. K., Shapiro, P. S., Rodig, S. R., Reddy, S. P., Platianis, L. C., Schreiber, R. D., and Kalvakolanu, D. V. (2001) J. Biol. Chem. 276, 287–297
34. Pandey, P. K., Udayakumar, T. S., Lin, X., Sharma, D., Shapiro, P. S., and Fendell, J. D. (2005) Mol. Cell. Biol. 25, 10695–10710
35. Ito, M., Yuan, C. X., Okano, H. J., Darnell, R. B., and Roeder, R. G. (2000) Mol. Cell 5, 683–693
36. Zhu, Y., Qi, C., Jia, Y., Nye, J. S., Rao, M. S., and Reddy, J. K. (2000) J. Biol. Cell. 725, 1283–1287
37. psychoactive medications (37). In summary, we show for the first time that Med1 plays a critical role in regulating C/EBP-β-driven IFN-induced transcription.
Chem. 275, 14779–14782
37. Ge, K., Guermah, M., Yuan, C. X., Ito, M., Wallberg, A. E., Spiegelman, B. M., and Roeder, R. G. (2002) Nature 417, 563–567
38. Lau, J. F., Nusinzon, I., Burakov, D., Freedman, L. P., and Horvath, C. M. (2003) Mol. Cell. Biol. 23, 620–628
39. Gordon, D. F., Tucker, E. A., Tundwal, K., Hall, H., Wood, W. M., and Ridgway, E. C. (2006) Mol. Endocrinol. 20, 1073–1089
40. Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L. C., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A., and Trouche, D. (1999) Biochem. Biophys. Res. Commun. 262, 157–162
41. Rowan, B. G., Garrison, N., Weigel, N. L., and O’Malley, B. W. (2000) Mol. Cell. Biol. 20, 8720–8730
42. Ait-Si-Ali, S., Ramirez, S., Barre, F. X., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J. A., Robin, P., Knibiehler, M., Pritchard, L. L., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998) Nature 396, 184–186
43. Misra, P., Owuor, E. D., Li, W., Yu, S., Qi, C., Meyer, K., Zhu, Y. J., Rao, M. S., Kong, A. N., and Reddy, J. K. (2002) J. Biol. Chem. 277, 48745–48754
44. Stevens, J. L., Cantin, G. T., Wang, G., Shevchenko, A., and Berk, A. J. (2002) Science 296, 755–758
45. Mo, X., Kowenz-Leutz, E., Xu, H., and Leutz, A. (2004) Mol. Cell 13, 241–250
46. Davis, J. A., Takagi, Y., Kornberg, R. D., and Asturias, F. A. (2002) Mol. Cell 10, 409–415
47. Taatjes, D. J., Marr, M. T., and Tjian, R. (2004) Nat. Rev. Mol. Cell. Biol. 5, 403–410
48. Taatjes, D. J., Schneider-Poetsch, T., and Tjian, R. (2004) Nat. Struct. Mol. Biol. 11, 664–671
49. Taatjes, D. J., and Tjian, R. (2004) Mol. Cell 14, 675–683
50. Hittelmann, A. B., Burakov, D., Iniguez-Lluhi, J. A., Freedman, L. P., and Garabedian, M. I. (1999) EMBO J. 18, 5380–5388
51. Yuan, C. X., Ito, M., Fondell, J. D., Fu, Z. Y., and Roeder, R. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7939–7944
52. Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997) J. Biol. Chem. 272, 25500–25506
53. Nevado, J., Tenbaum, S. P., and Aranda, A. (2004) Mol. Cell. Endocrinol. 222, 41–51
54. Malik, S., Wallberg, A. E., Kang, Y. K., and Roeder, R. G. (2002) Mol. Cell. Biol. 22, 5626–5637
55. Park, J. M., Kim, J. M., Kim, I. K., Kim, S. N., Kim-Ha, J., Kim, J. H., and Kim, Y. J. (2003) Mol. Cell. Biol. 23, 1358–1367
56. Kim, T. W., Kwon, Y. J., Kim, J. M., Song, Y. H., Kim, S. N., and Kim, Y. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12153–12158
57. Ito, M., Yuan, C. X., Malik, S., Gu, W., Fondell, J. D., Yamamura, S., Fu, Z. Y., Zhang, X., Qin, J., and Roeder, R. G. (1999) Mol. Cell 3, 361–370
58. Drane, P., Barel, M., Balbo, M., and Frade, R. (1997) Oncogene 15, 3013–3024
59. Frade, R., Balbo, M., and Barel, M. (2000) Cancer Res. 60, 6585–6589
60. Kowenz-Leutz, E., and Leutz, A. (1999) Mol. Cell 4, 735–743
61. Kowenz-Leutz, E., Twamley, G., Ansideau, S., and Leutz, A. (1994) Genes Dev. 8, 2781–2791