The Transcriptional Coactivator CREB-binding Protein Cooperates with STAT1 and NF-κB for Synergistic Transcriptional Activation of the CXC Ligand 9/Monokine Induced by Interferon-γ Gene*

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Signal transducers and activators of transcription 1 (STAT1) and NF-κB cooperatively regulate the expression of many inflammatory genes. In the present study, we demonstrate that the transcriptional coactivator CREB-binding protein (CBP) mediated the STAT1/NF-κB synergy for transcription of the gene for CXCL9, an interferon-γ (IFN-γ)-inducible chemokine. Reporter gene analysis showed that expression of CBP potentiated IFN-γ and tumor necrosis factor (TNFα)-induced promoter activity and that the CBP-mediated synergy depended upon STAT1- and NF-κB-binding sites in the promoter. Experiments with CBP mutants indicated that the N-terminal and C-terminal regions were necessary for the transcriptional synergy, although the histone acetyltransferase activity of CBP was dispensable. A co-immunoprecipitation assay demonstrated that STAT1 and NF-κB RelA (p65) simultaneously associated with CBP in vivo. Furthermore, chromatin immunoprecipitation revealed that, although costimulation with IFN-γ and TNFα did not cooperatively enhance the levels of acetylated histones, it did result in increased recruitment of STAT1, CBP, and RNA polymerase II at the promoter region of the CXCL9 gene. Together, these results demonstrate that the STAT1/NF-κB-dependent transcriptional synergy could result from the enhanced recruitment of RNA polymerase II complex to the promoter via simultaneous interaction of CBP with STAT1 and NF-κB.

Control of immune responses and inflammatory reaction is mediated by intercellular communication through direct cell-to-cell interactions and soluble factors such as cytokines. Cytokine-mediated intercellular communication is often orchestrated through cross-talk between different classes of cytokines and extracellular stimuli. Interferon-γ (IFN-γ) promotes the development of cell-mediated immunity and functions cooperatively with other extracellular stimuli such as tumor necrosis factor-α (TNFα) or lipopolysaccharide to induce the expression of a number of proinflammatory genes including major histocompatibility complex class I (1), inducible nitric-oxide synthase (2, 3), intercellular adhesion molecule 1 (4), and interferon-inducible chemokine CXCL10/IFN-inducible protein of 10 kDa (5).

Cytokine-mediated transcriptional activations of inflammatory genes has been studied extensively. NF-κB plays critical roles in transcriptional regulation of numerous genes involved in host-defense mechanisms (6). Prototypically, the NF-κB1 (p50)/RelA (p65) heterodimer is sequestered in the cytoplasm by inhibitor protein IκB. Upon stimulation with extracellular signals such as proinflammatory cytokines or bacterial or viral components, IκB is phosphorylated by IκB kinases, ubiquitinated, and degraded by 26 S proteasomes. After degradation of IκB, NF-κB is translocated to the nucleus and binds to κB elements found in many inflammatory genes (7). Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors that are phosphorylated at a single tyrosine residue via members of the Jak kinase family following stimulation with cytokines, hormones, or growth factors; are assembled in dimeric form; are translocated to the nucleus; and become bound to specific DNA sequence motifs (8–10). IFN-γ activates the STAT1α homodimer that binds to IFN-γ-activation sequences (11) found in the promoter regions of a number of IFN-γ-inducible genes including interferon regulatory factor 1 (12) and chemokine CXCL9, which is a monokine induced by IFN-γ (MIG) (13, 14). The NF-κB- and STAT-dependent signaling pathways are integral to the transcriptional regulation of many inflammatory genes, and these transcriptional factors often cooperatively regulate the transcriptional activation of many genes (4, 5, 15, 16). Previous studies have demonstrated that IFN-γ-induced STAT1α and TNFα-induced NF-κB synergistically regulate the transcription of the intercellular adhesion molecule-1 and IRF-1 genes (4, 15, 16), although the molecular mechanisms involved in the STAT1α/NF-κB-mediated transcriptional cooperation remain to be elucidated.

Transcriptional coactivator CREB-binding protein (CBP) and closely related p300 play a critical role in various aspects of transcriptional regulation (17–19). One of the major functions of coactivator CBP/p300 is to function as a bridging factor between sequence-specific transcriptional activator and basal transcriptional machinery and to assemble them to form a stable multiprotein complex. CBP/p300 also possesses an intrinsic histone acetyltransferase (HAT) activity, which modifies the histone tail to destabilize the chromatin structure and thus increase the accessibility of the basal transcriptional ma...
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...expression plasmids by using Polyfect transfection reagents (Qiagen, Valencia, CA) according to the manufacturer's instructions. For standardization of the transfection efficiencies for the luciferase reporter assay, the transfected cells were harvested, pooled, and seeded in 24-well culture plates. After 24 h, the cells were treated with IFN-γ and/or TNFα for 8 h. Firefly and Renilla luciferase activities were assayed by using reagents provided by Promega according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as described previously (5) by use of a modification of the method of Dignam et al. (38). The following oligonucleotides were used in the EMSA: 5'-RE, 5'-GATCCCTTAAGTTAAAAAGCCATTTCTGATGTTC-3'; 5'-RE, 5'- GATCCCTTAAGTTAAAAAGCCATTTCTGATGTTC-3'; 5'-RE, 5'-GATCCCTTAAGTTAAAAAGCCATTTCTGATGTTC-3'; 5'-RE, 5'-GATCCCTTAAGTTAAAAAGCCATTTCTGATGTTC-3'. Binding reactions and antibody supershift assays were described previously (5, 15).

Immunoprecipitation and Western Blot Analysis—Cells were washed with ice-cold phosphate-buffered saline, harvested, resuspended in lysis buffer (50 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, 2.5 mM MgCl2, 0.5% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonfyl fluoride, and 10 μg/ml each of leupeptin, antipain, aprotinin, and pepstatin), and kept on ice for 10 min. After precipitation of the lysate, the whole cell lysate (~500 μg of protein) was incubated with anti-V5 (1 μg) antibody or normal mouse IgG and protein G-Sepharose (50% slurry) for 16 h at 4°C. The immunoprecipitates were washed four times with 1 ml of lysis buffer, eluted with SDS-PAGE sample buffer, resolved on 7.5% SDS-PAGE, and analyzed by Western blotting.

Immunocytochemistry—Cells grown on Lab-tek chamber slides (Nunc, Rochester, NY) were fixed at room temperature in 4% paraformaldehyde and 0.5% Triton X-100. They were then reacted with mouse anti-V5 antibody at room temperature for 1 h, and unbound antibody was subsequently removed by washing with phosphate-buffered saline. Bound antibody was detected with goat anti-mouse IgG conjugated with Alexa (488 nm) (Molecular Probes). Immunofluorescence was detected by confocal laser-scanning microscopy (LSM 510, Carl Zeiss, Goettingen, Germany).

CAIP—ChIP was performed as described previously (39, 40) with some modification. Briefly, confluent monolayers of NIH3T3 cells were fixed with formaldehyde (1% v/v) overnight at 4°C. Following cross...
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FIG. 2. The γRE and xB sites in the promoter region of the CXCL9 gene are required for IFN-γ- and TNFα-induced transcriptional synergy. A, schematic representation of wild-type and mutant Mig-328 luciferase reporter constructs. B, HEK293 cells were transiently co-transfected with either empty vector or the CBP expression plasmid (2 μg) and the indicated wild-type or mutant Mig-328 luciferase reporter construct (1 μg). 24 h after transfection, the cells were either left untreated or treated with IFN-γ and/or TNFα (10 ng/ml each) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as -fold induction compared with the activity of the unstimulated control. C, individual NIH3T3 cell cultures were transiently co-transfected with a luciferase reporter construct containing 328 bp of a 5'-flanking sequence of the CXCL9 gene and an expression vector encoding CBP or empty vector. After transfection, the cells were stimulated with IFN-γ and/or TNFα or left untreated before analysis of the luciferase reporter gene activity. As shown in Fig. 1, although IFN-γ and TNFα alone had only a minimum effect on the CXCL9 promoter activity in the absence of CBP, cotransfection with IFN-γ and TNFα synergistically induced the promoter activity. When the cells were co-transfected with the expression vector encoding CBP, the cooperative response to IFN-γ and TNFα was further potentiated. This result indicates that CBP functioned to mediate the synergy between IFN-γ and TNFα for the transcription of the CXCL9 gene.

γRE and xB2 Sites Are Required for IFN-γ- and TNFα-induced Transcriptional Synergy—In previous studies, we and others showed that both a STAT1 binding element and NF-κB binding site were required for the IFN-γ- and TNFα-induced transcriptional synergy (4, 5, 15, 16). There are several potential regulatory elements in the promoter region of the CXCL9 gene (Fig. 2A). Although γRE has been identified as an IFN-γ-responsive site that is recognized by the STAT1 tetramer (14, 31), the functional significance of several putative xB sites within –200 bp of the promoter has not been analyzed. To determine the regulatory elements responsible for the CBP-mediated transcriptional synergy, we mutated the γRE and these putative xB sites and analyzed the mutant ones for their possible role in IFN-γ- and TNFα-induced promoter activity. As
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modestly increased the binding activity of complex II (lane 2), but co-stimulation with IFN-γ and TNFα did not enhance these DNA binding activities (lane 4). The antibody supershift assay demonstrated that complexes I and II contained STAT1 (Fig. 3B, lane 8), which correspond to the previously identified γRF-1 and γRF-2, respectively (14, 31). Antibody to NF-κB1 (p50) and RelA (p65) also reduced the binding activity of complexes II and III (lanes 9–11), indicating that complexes II and III contained NF-κB1 (p50)/RelA (p65) and NF-κB1 (p50) homodimer, respectively. In this regard, NF-κB was previously reported to bind to the γRE of the CXCL9 gene as well as to the IFN-γ activation sequence of the IRF-1 gene (15, 16, 31). In our EMSA study using the κB motifs from the promoter region of the CXCL9 gene as probes, a marked DNA binding activity was observed at the κB2 motif (Fig. 3C, lanes 5–8), consistent with the result of the promoter analysis showing that mutation of the κB2 site significantly reduced the promoter activity (Fig. 2). When the cells were costimulated with IFN-γ and TNFα, the binding activity toward these κB motifs was unchanged (Fig. 3C, lanes 4, 8, and 12). The antibody supershift assay showed that NF-κB2 (p50)/RelA (p65) and NF-κB1 (p50) homodimer bound to the κB2 motif (Fig. 3D). Thus, these results indicate that STAT1 and NF-κB had the ability to bind to these sites independently.

STAT1 and RelA Simultaneously Interact with the N- and C-terminal Regions of CBP in Vivo—Because STAT1 and NF-κB have been shown to interact with CBP independently (24–28), synergistic transcriptional activity of the CXCL9 promoter by IFN-γ/STAT1 and TNFα/NF-κB could result from the simultaneous physical interaction of CBP with STAT1 and NF-κB. To determine whether CBP simultaneously associates with STAT1 and NF-κB in vivo, we performed communoprecipitation experiments (Fig. 4). HEK293 cells were co-transfected with an expression plasmid encoding the N-terminal (amino acids 1–777) or the C-terminal region of CBP (amino acids 1758–2441) together with STAT1 and RelA expression vectors. These transfected mutants resided in the nucleus, as shown by immunostaining (Fig. 4B). After stimulation with IFN-γ and/or TNFα, whole cell lysates were prepared, immunoprecipitated with antibody against the V5 epitope tag, and assessed by Western blotting with antibody against STAT1. After detection of STAT1, the blots were stripped and reprobed with antibody against RelA. As shown in Fig. 4C (lanes 5–8), whereas both STAT1 and RelA were constitutively detected in lysates immunoprecipitated with the N-terminal region of CBP 1–777, the association of CBP with RelA was enhanced by TNFα stimulation (lanes 7 and 8). Immunoprecipitates of lysates from the C-terminal region (CBP 1758–2441)-transfected cells also constitutively contained STAT1 and RelA (p65) (lane 13), although the interaction with STAT1 was enhanced by IFN-γ stimulation (lanes 14 and 16). We also generated a deletion mutant CBP 775–1779NLS lacking both the N-terminal and C-terminal regions of CBP (Fig. 4A). This construct contained a nuclear localization signal from the SV40 T antigen, since the original construct did not translocate to the nucleus. Although the CBP 775–1779NLS resided in both the cytoplasm and the nucleus (Fig. 4B), no associations with STAT1 and RelA were observed under unstimulated or stimulated conditions (Fig. 4E), indicating that the N-terminal and C-terminal regions of CBP specifically interacted with STAT1 and RelA. Thus, these results indicate that CBP is capable of interacting with STAT1 and NF-κB simultaneously. Furthermore, the nature of the interaction between CBP and STAT1 or NF-κB appeared to be distinct; i.e. IFN-γ-activated STAT1 preferentially interacted with the C-terminal region of CBP, and TNFα-stimulated NF-κB RelA

shown in Fig. 2B, mutation of 5′-γRE or 3′-γRE abolished the cooperative response to IFN-γ and TNFα without affecting the TNFα-induced luciferase activity (lanes 3 and 4). Whereas mutation of the κB1 site had little effect on the promoter activity (lane 5), mutation of the κB3 site diminished the cooperative response to IFN-γ and TNFα (lane 7). Furthermore, mutation of the κB2 site abolished the response to TNFα and markedly reduced the cooperativity for IFN-γ and TNFα (lane 6). Mutation of both the 3′-γRE and κB2 sites almost completely eliminated the sensitivity to both stimuli (lane 9). The requirement of the γRE and κB2 sites for mediating the IFN-γ and TNFα-induced transcriptional synergy was also observed in NIH3T3 cells (Fig. 2C). Taken together, these results indicate that both the γRE and κB2 sites are required to mediate the transcriptional synergy of the CXCL9 gene.

γRE and κB DNA Binding Activities in Nuclear Extracts from IFN-γ- and TNFα-stimulated Cells—An EMSA study was carried out to examine the γRE and κB DNA binding activities in nuclear extracts from IFN-γ- and/or TNFα-stimulated cells. As seen in Fig. 3A, IFN-γ induced formation of complex I and

FIG. 3. γRE and κB DNA binding activities in nuclear extracts from IFN-γ- and TNFα-stimulated cells. NIH3T3 were either left untreated or treated with IFN-γ and TNFα (10 ng/ml each) for 30 min before the preparation of nuclear extracts. 10 μg of each nuclear extract was analyzed for γRE (A and B) or κB (C and D) binding activity by EMSA using radiolabeled oligonucleotides as described under “Experimental Procedures.” In some experiments (B and D), nuclear extracts were incubated with the indicated antibodies (1 μg) before analysis of the binding activities. Supershifted complexes (s.s.) and nonspecific binding (ns) are indicated. Similar results were obtained from three separate experiments.

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interacted with the N-terminal region of CBP.

FIG. 4. STAT1 and RelA simultaneously interact with the N- and C-terminal regions of CBP in vivo. A, the diagram shows the structure of wild-type CBP including its functional domains and those structures of N-terminal and C-terminal truncated mutant constructs. Proteins known to interact with CBP are indicated at the top of the diagram. Numbers denote amino acid positions. RID, receptor-interacting domain; CH, cysteine-histidine-rich region; KIX, kinase-induced interaction domain; Bromo, bromodomain; IBiD, IRF-3 binding domain. CBP 775–1779 contained an NLS from SV40 in front of the V5 epitope tag. B, immunofluorescence microscopy of HEK293 cells transfected with CBP mutants. HEK293 cells transfected with V5 epitope-tagged deletion mutants were fixed and labeled with anti-V5 antibody. Immunofluorescence staining was detected by confocal laser-scanning microscopy. C and E, HEK293 cells were transiently co-transfected with V5-epitope-tagged expression plasmid together with STAT1 and RelA expression vectors. 24 h after transfection, the cells were either left untreated or treated with IFN-γ and/or TNFα (10 ng/ml each) for 1 h. Whole cell extracts were prepared, immunoprecipitated (IP) with anti-V5 antibody or normal mouse IgG as indicated, and analyzed by Western blotting using anti-STAT1 antibody. The blots were then stripped and analyzed with anti-RelA and again stripped and treated with anti-V5 antibodies. D, whole cell extracts prepared as described above were assessed for STAT1 and RelA by Western blotting using ant-STAT1 and anti-RelA antibodies.

Synergy—To determine the functional significance of these interactions for mediating the transcriptional synergy, we assessed the N- and C-terminal deletion mutants of CBP for their
transactivating function with respect to the CXCL9 promoter (Fig. 5). Although progressive C-terminal deletions to amino acid residue 2027 had little effect on the IFN-γ/STAT1 and TNFα/NF-κB-mediated transcriptional synergy, deletion to amino acid 1779 abolished the synergistic transcription, indicating that the region between residues 2027 and 1779 was required to mediate the transcriptional synergy. This region contains cysteine-histidine-rich domain 3, which is known to interact with STAT1 as well as with RNA helicase A and RNA polymerase II (24, 25, 43). Transfection with the N-terminal deletion mutant (CBP 450–2441), which retains the CREB-binding domain KIX, also reduced the IFN-γ/STAT1 and TNFα/NF-κB-mediated transcriptional synergy. These results demonstrate that both the N-terminal 450 amino acids and cysteine-histidine-rich domain 3 of CBP are required to mediate the IFN-γ/STAT1 and TNFα/NF-κB-induced transcriptional synergy.

Previous studies have shown that STAT1 and RelA/p65-dependent transcription require coactivator NcoA-3 (p/CIP) and NcoA-1 (SRC-1), respectively, which interact with the region between residues 2058 and 2163 of CBP (44, 45), and this region has been recently identified as the IRF-3 binding domain (IbiD) (46). Interestingly, whereas the deletion mutant CBP 1–2027, which lacks the IbiD, was capable of mediating the synergistic response, the TNFα-induced promoter activity was significantly reduced. This result is consistent with the previous finding that RelA/p65-dependent transcription requires the NcoA-1 (SRC-1) interacting domain of CBP (45). Together, these results suggest that although IbiD is required for individual RelA/p65- or STAT1-dependent transcription, the IFN-γ/STAT1- and TNFα/NF-κB-mediated transcriptional synergy does not require this region.

The IFN-γ/STAT1 and TNFα/NF-κB-induced Transcriptional Synergy Does Not Require the HAT Activity of CBP—CBP possesses an intrinsic HAT domain that regulates the transcriptional activities of various transcription factors (20–22). In order to determine whether the HAT activity of CBP was required for the IFN-γ/STAT1 and TNFα/NF-κB-induced transcriptional synergy, we tested an expression construct containing a mutant HAT domain for its transactivating function. The mutation of the HAT domain used here was previously demonstrated to abolish the HAT activity (33). As shown in Fig. 6, the mutant construct (CBP mHAT) was able to enhance the promoter activity in response to IFN-γ and/or TNFα (10 ng/ml each) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as -fold induction compared with the activity of unstimulated samples. Each column and bar represents the mean ± S.E. of three independent experiments.

Fig. 5. The N-terminal and C-terminal regions of CBP are required for the STAT1 and NF-κB-dependent transcriptional synergy. A, the diagram shows wild-type CBP and N-terminal and C-terminal deletion mutants. Proteins known to interact with CBP are indicated, as defined in the legend to Fig. 4. B, HEK293 cells were transiently co-transfected with either empty vector or the wild-type or the mutant CBP expression plasmid (2 μg) and Mig-328 luciferase reporter construct (1 μg). 24 h after transfection, the cells were either left untreated or treated with IFN-γ and/or TNFα (10 ng/ml each) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as -fold induction compared with the activity of unstimulated samples. Each column and bar represents the mean ± S.E. of three independent experiments.

Fig. 6. CBP HAT activity is dispensable for the IFN-γ/STAT1- and TNFα/NF-κB-induced transcriptional synergy. HEK293 cells were transiently co-transfected with either empty vector or either of the CBP expression plasmids (2 μg) as indicated and the Mig-328 luciferase reporter construct (1 μg). 24 h after transfection, the cells were either left untreated or treated with IFN-γ and/or TNFα (10 ng/ml each) for 8 h prior to measurement of luciferase activity. The relative luciferase activity is shown as -fold induction compared with the activity of unstimulated samples. Each column and bar represents the mean ± S.E. of three independent experiments.
Costimulation with IFN-γ and TNFα Does Not Induce Histone Hyperacetylation at the CXCL9 Promoter—It was earlier demonstrated that highly acetylated histone correlates with transcriptionally active chromatin, which facilitates recruitment of the basal transcriptional machinery (22). Although the intrinsic HAT activity of CBP is dispensable for the IFN-γ/STAT1- and TNFα/NF-κB-induced transcriptional synergy, CBP interacts with other coactivators such as p/CAF, NcoA1 (SRC-1), and NcoA3 (p/CIP/ACTR), which also possess HAT activity (47–49). To determine whether histone hyperacetylation could be one of the mechanisms for the transcriptional synergy, we assessed the acetylation status of the promoter region of the CXCL9 gene in NIH3T3 cells by using a ChIP assay (Fig. 7). Initially, we monitored the acetylation status of the CXCL9 promoter in the presence of trichostatin A (TSA), a histone deacetylase inhibitor (50). Whereas a relatively low basal level of histone H4 acetylation was observed at the promoter in untreated cells (Fig. 7B, lane 6), treatment of cells with TSA led to a time-dependent increase in the amount of acetylated histone H4 (lanes 7–10), indicating that the promoter region of the CXCL9 gene is deacetylated by TSA-sensitive histone deacetylase with the cells in the quiescent state. To determine whether costimulation with IFN-γ and TNFα led to hyperacetylation at the CXCL9 promoter, we performed a ChIP assay with antibodies against anti-acetylated histones H3 and H4 (Fig. 7C). Treatment of cells with IFN-γ or TNFα for 4 h induced a significant increase in acetylated histone H3 (Fig. 7C, lanes 7 and 8). However, there was no further increase in the level of acetylated histone in IFN-γ- and TNFα-treated cells (lane 9) despite the fact that a marked synergism between IFN-γ and TNFα was observed in the expression of the endogenous CXCL9 gene (Fig. 7D, lane 4). Interestingly, whereas the level of acetylated histone H4 was also enhanced in IFN-γ-stimulated cells (Fig. 7C, lane 7), TNFα only modestly stimulated the acetylation (lane 8), and no cooperative effect on the histone acetylation was observed in IFN-γ- and TNFα-treated cells (lane 9). Thus, these results indicate that IFN-γ STAT1 and TNFα/NF-κB-induced transcriptional synergy with respect
to the CXCL9 gene does not correlate with histone hyperacetylation at the promoter.

Costimulation with IFN-γ and TNFα Cooperatively Recruits STAT1, CBP, and RNA Polymerase II to the CXCL9 Promoter—

Next, by using the ChIP assay, we examined whether costimulation with IFN-γ and TNFα could induce a cooperative binding of STAT1 and/or NF-κB to the CXCL9 promoter (Fig. 8A). Although IFN-γ alone modestly recruited STAT1 to the promoter (lane 6), costimulation with IFN-γ and TNFα led to an increase in occupancy of STAT1 (lane 8). We were, however, unable to detect significant occupancy of NF-κB at the promoter region. It is possible that antigen determinants of RelA might be masked by the multiple protein complex bound to the promoter.

Because STAT1 interacts with CBP, the cooperative binding of STAT1 may lead to an increased recruitment of CBP at the promoter. Furthermore, since cysteine-histidine-rich domain 3 (1805–1890 amino acids) of CBP was required for the transcriptional synergy (Fig. 5) and this domain has been demonstrated to interact with RNA polymerase II (RNA Pol II) via RNA helicase A (43), it is conceivable that CBP recruits RNA Pol II to the CXCL9 promoter in response to IFN-γ/STAT1 and TNFα/NF-κB. To test these possibilities, we next assessed the recruitment of CBP and RNA Pol II to the CXCL9 promoter by using the ChIP assay. As shown in Fig. 8B, whereas IFN-γ and TNFα alone had only minimal effect on the recruitment of CBP (lanes 6 and 7), costimulation with IFN-γ and TNFα cooperatively recruited CBP to the promoter (lane 8). Consistent with this, a marked increase in the recruitment of RNA Pol II to it was observed in IFN-γ- and TNFα-treated cells. Thus, taken together, these results indicate that the IFN-γ/STAT1- and TNFα/NF-κB-induced transcriptional synergy is, at least partially, mediated by recruiting RNA Pol II to the promoter region of the CXCL9 gene via CBP.

**DISCUSSION**

Transcription of the genes that contain STAT1-binding elements and NF-κB-binding sites in their promoter regions are often cooperatively regulated by extracellular stimuli that induce STAT1 and NF-κB, such as IFN-γ and TNFα or bacterial lipopolysaccharide (2–5, 15, 16). We and others previously reported that IFN-γ-induced STAT1α and TNFα-induced NF-κB synergistically regulated the transcription of many inflammatory genes (4, 5, 15, 16). Although independent interaction of STAT1 and NF-κB with their cognate binding sites was shown to be sufficient for mediating the transcriptional synergy (15), the molecular mechanisms involved in the STAT1- and NF-κB-mediated transcriptional synergy remained to be elucidated. In the present study, we evaluated the potential role of coactivator CBP in the control of the transcriptional synergy between IFN-γ/STAT1 and TNFα/NF-κB. Our results demonstrate that simultaneous interactions of CBP with IFN-γ-induced STAT1 and TNFα-activated NF-κB RelA (p65) were required to mediate the transcriptional synergy. Furthermore, the IFN-γ/STAT1- and TNFα/NF-κB-induced transcriptional synergy appears to be mediated by increased recruitment of RNA polymerase II to the promoter region of the CXCL9 gene via CBP. These conclusions are based on the following observations. 1) Overexpression of CBP potentiated IFN-γ/STAT1- and TNFα/NF-κB-induced cooperative transcriptional activation of the CXCL9 gene. 2) The CBP-mediated synergistic transcriptional activity of the CXCL9 promoter was abolished by mutation of the yRE and the κB sites. 3) The N-terminal 450 residues and the C-terminal region between amino acids 2027 and 1779 were required for the CBP-mediated transcriptional synergy. 4) IFN-γ-induced STAT1 and TNFα-activated NF-κB RelA (p65) simultaneously interacted with distinct regions of CBP. 5) Costimulation with IFN-γ and TNFα cooperatively recruited STAT1, CBP, and RNA Pol II to the promoter region of the CXCL9 gene.

One of the mechanisms involved in the IFN-γ/STAT1 and TNFα/NF-κB-induced transcriptional synergy appears to be the enhanced recruitment of RNA Pol II to the promoter region of the CXCL9 gene. Since CBP has been shown to associate with RNA Pol II via RNA helicase A (43), the simultaneous interaction of CBP with STAT1 and NF-κB might stabilize the binding of CBP to the promoter, and the stabilized CBP could provide a stable scaffold for the RNA polymerase II complex. Indeed, our data from the ChIP assay (Fig. 8) demonstrated that co-treatment with IFN-γ and TNFα led to an increase in the recruitment of CBP and RNA Pol II to the CXCL9 promoter. The data from the ChIP assay also demonstrated that the costimulation with IFN-γ and TNFα induced a cooperative occupancy of STAT1 to the promoter. Although we were unable to obtain data for the ChIP assay of NF-κB, STAT1 and NF-κB bound to the CXCL9 promoter may create an enhanceosome-like structure that leads to the cooperative recruitment of CBP/Pol II to the promoter (51). Although STAT1 and NF-κB have been shown to interact with other components of the transcriptional machinery (52, 53), the role of these factors in the IFN-γ/STAT1- and TNFα/NF-κB-induced transcriptional synergy remains to be determined.

STAT1 and NF-κB (RelA/p65) were previously shown to bind to the N-terminal and the C-terminal regions of CBP (24–28). We confirmed these physical interactions in vivo and extended our observations to show that STAT1 and NF-κB were capable of interacting with CBP simultaneously to form a trimeric complex. Although both the N- and C-terminal regions of CBP have the capacity to interact with STAT1 and NF-κB, the
nature of the physical association appears to be different. The interaction of STAT1 with the C-terminal region of CBP was enhanced by IFN-γ treatment (Fig. 3), suggesting that tyrosine-phosphorylated STAT1 preferentially interacted with the C-terminal region of CBP. In this regard, the C-terminal activation domain of STAT1 has been shown to bind to cysteine-histidine-rich domain 3 of CBP (24). In contrast, the association of STAT1 with the N-terminal region of CBP was constitutive and much weaker than the C-terminal interaction. Thus, it is likely that the C-terminal interaction of CBP with STAT1 may participate in mediating the transcriptional synergy in response to IFN-γ. We did not, however, detect stimulus-dependent interaction of STAT1 with the N-terminal region of CBP, as was previously reported (24, 25). Although the reason for this difference is currently unclear, the difference in expression systems might be a possible explanation. In addition to the interaction of STAT1 with the C-terminal part of CBP, interaction of RelA with the N-terminal region (amino acids 1–450) of CBP appeared to be required for mediating the transcriptional synergy. As shown in Figs. 4 and 5, although RelA had the ability to interact with both the N-terminal and C-terminal region of CBP, deletion of the N-terminal 450 amino acid residues but not the C-terminal deletion to 2027 residues abolished the transcriptional synergy. Furthermore, the interaction of RelA with the N-terminal region of CBP was enhanced by TNFα treatment (Fig. 4), suggesting that nuclear translocated RelA associates with the N-terminal region of CBP. The N-terminal region of CBP (amino acids 1–450) has been shown to interact with the transactivating domain of RelA (amino acids 313–550) (27, 28). Thus, simultaneous interaction of CBP with IFN-γ-activated STAT1α through the C-terminal region of CBP and with TNFα-activated RelA through its N-terminal region is likely to mediate the transcriptional synergy of the CXCL9 gene.

The requirement for the coactivator NcoA-3 (p/CIP) and NcoA-1 (SRC-1) interacting region of CBP, which has been recently identified as the IRF-3 binding domain (46), to mediate the STAT1/NF-κB-induced transcriptional synergy appears to be different from that for individual STAT1 or NF-κB RelA/p65-dependent transcription. Although previous studies have shown that STAT1 and NF-κB RelA/p65-dependent transcription require coactivator NcoA-3 (p/CIP) and NcoA-1 (SRC-1), respectively (44, 45), as shown in this study, the C-terminal deletion mutant (CBP 1–2027), which lacked the IRF-3 binding domain, was able to mediate the STAT1/NF-κB-induced transcriptional synergy shown for the CXCL9 promoter (Fig. 5). This suggests that some other region(s) of CBP may compensate for the transactivating function or that the requirement for NcoA-3 (p/CIP) and NcoA-1 (SRC-1) to mediate the transcriptional activation may depend upon the promoter context.

Although HAT-dependent and -independent transcriptional activations have been demonstrated for various transcriptional factors in different promoter contexts (22, 33, 35, 45, 54), the IFN-γ/STAT1 and TNFα/NF-κB-induced transcriptional synergy of the CXCL9 gene did not require the CBP HAT activity (Fig. 6). This conclusion was further supported by the finding that costimulation with IFN-γ and TNFα did not increase the levels of histone acetylation at the CXCL9 promoter (Fig. 7). The level of acetylated histone at the promoter in the cells treated with IFN-γ and TNFα was comparable with that in TSA treatment, a histone deacetylase inhibitor (50), suggesting that the promoter region is highly acetylated. This result suggests that although CBP has been shown to interact with other histone acetylases such as pCAF, NcoA-1 (SRC-1), and NcoA-3 (p/CIP/ACTR) (47–49), the histone hyperacetylation at the promoter region may not be the primary mechanism for the transcriptional synergy of the CXCL9 gene. Furthermore, histone acetylation per se is not sufficient for mediating transcriptional activation of the CXCL9 gene. As seen in the ChIP assay (Fig. 7C), although treatment of cells with IFN-γ or TNFα alone markedly acetylated the promoter region, either stimulus alone did not significantly induce the transcriptional activation of the CXCL9 gene (Figs. 2C and 7D). Taken together, these results indicate that the histone acetylation at the CXCL9 promoter may be necessary for some step in the transcription but is not sufficient for mediating the transcriptional activation of the CXCL9 gene. The requirement of histone acetylation for STAT1/NF-κB-dependent transcriptional synergy in other genes remains to be determined.

STAT1 and NF-κB are integral transcription factors functioning in the regulation of genes involved in immune and inflammatory reactions. Activations of STAT1 and NF-κB are normally induced by distinct classes of extracellular signals present in the microenvironment. Type I and type II IFNs activate STAT1, whereas members of the TNF family and ligands for Toll-like receptors including lipopolysaccharide induce activation of NF-κB. When the cells are exposed to stimuli that activate both signaling pathways, this could ultimately promote type I immune responses, which are associated with host-defense mechanisms against viral and bacterial infections and excessive immune response that could result in some type of autoimmune disease (55, 56). Our study presented here provides an insight into the molecular mechanisms involved in the interplay between STAT1 and NF-κB to control the synergistic transcriptional activation of the inflammatory genes associated with type I responses.
CBP Mediates Transcriptional Synergy between NF-κB and STAT1

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The Transcriptional Coactivator CREB-binding Protein Cooperates with STAT1 and NF-κB for Synergistic Transcriptional Activation of the CXC Ligand 9/Monokine Induced by Interferon-γ Gene

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