The Transcriptional Coactivator CBP Cooperates with STAT1 and NF-kB for Synergistic Transcriptional Activation of the CXCL9/MIG Gene

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Running Title:

CBP Mediates Transcriptional Synergy between NF-κB and STAT1
Summary

Signal transducers and activators of transcription 1 (STAT1) and nuclear factor κB (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 CXC ligand 9 (CXCL9), an interferon gamma (IFNγ)-inducible chemokine. Reporter gene analysis showed that expression of CBP potentiated IFNγ and tumor necrosis factor (TNFα)-induced promoter activity and that the CBP-mediated synergy depended upon a 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. 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 IFNγ 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.
Introduction.

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 crosstalk between different classes of cytokines and extracellular stimuli. Interferon gamma (IFNγ) promotes the development of cell-mediated immunity and functions cooperatively with other extracellular stimulus such as tumor necrosis factor α (TNFα) or lipopolysaccharide (LPS) to induce the expression of a number of pro-inflammatory genes including MHC class I (1), inducible nitric oxide synthase (2,3), intercellular adhesion molecule 1 (ICAM-1, ref. 4), and interferon-inducible chemokine CXC ligand 10 (CXCL10)/IFN inducible protein 10 kDa (IP-10, ref. 5).

Cytokine-mediated transcriptional activations of inflammatory genes has been studied extensively. Nuclear factor κB (NF-κB) plays critical roles in transcriptional regulation of numerous genes involved in host-defense mechanisms (6). Prototypically, the NFκB1 (p50)/RelA (p65) heterodimer is sequestered in the cytoplasm by inhibitor protein IκB. Upon stimulation with extracellular signals such as pro-inflammatory cytokines or bacterial or viral components, IκB is phosphorylated by IκB kinases, ubiquitinated, and degradated by 26S 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, assembled in dimeric form, translocated to the nucleus, and become bound to specific DNA sequence motifs (8-10). IFNγ activates the STAT1α homodimer that binds to IFNγ-activation sequences (GAS, ref. 11) found in the promoter region of a number of IFNγ-inducible genes including interferon regulatory factor 1 (IRF-1, ref. 12) and chemokine CXCL9, which is a monokine induced by IFNγ (MIG, refs. 13,14). The NF-κB- and STAT-
dependent signalling 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 IFNγ-induced STAT1α and TNFα-induced NF-κB synergistically regulate the transcription of the ICAM-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 multi-protein 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 machinery to the DNA template (20-22). Furthermore, HAT alters the activities of a number of non-histone transcription factors such as p53 by acetylating them, and thereby stimulates their DNA binding and transcriptional activities (23).

Because CBP has been shown to function as a coactivator for STAT1 and NF-κB (24-28), we hypothesized that CBP might play an essential role in the transcriptional synergy between STAT1 and NF-κB in inflammatory gene expression. In this presently reported study we explored the mechanisms through which CBP integrates the crosstalk between IFNγ/STAT1 and TNFα/NF-κB signaling pathways to cooperatively induce the transcriptional activation of the gene for CXCL9, an IFNγ-inducible chemokine (13,29). The results presented in this study demonstrate that CBP mediates the transcriptional synergy between IFNγ/STAT1 and TNFα/NF-κB in the CXCL9 gene. Whereas the HAT activity of CBP was dispensable for the synergy, the N-terminal 450 residues and C-terminal region between 1779 and 2027 amino acids were required to mediate the
transcriptional synergy. Consistent with this and extending prior studies, STAT1 and NF-κB were shown to simultaneously interact with the N- and C-terminal regions of CBP. Furthermore, the results of a chromatin immunoprecipitation (ChIP) assay demonstrated that IFNγ and TNFα cooperatively recruited STAT1 and CBP-RNA polymerase II to the promoter region of the CXCL9 gene. These results indicate that CBP mediates the IFNγ/STAT1 and TNFα/NF-κB-induced transcriptional synergy by recruiting the RNA polymerase II complex to the CXCL9 promoter via simultaneous interaction with STAT1 and NF-κB.
Experimental Procedures

Reagents and Antibodies

Recombinant IFNγ and TNFα were obtained from R and D Systems Inc (Minneapolis, MN). Antibodies against STAT1, CBP, NF-κB p50, RelA(p65), and RNA polymerase II were obtained from Santa Cruz Biotechnology (Hercules, CA); and anti-acetylated-histone H3 and H4, from Upstate Biotechnology (Lake Placid, NY). Anti-V5 epitope tag antibody came from Invitrogen (Carlsbad, CA). Goat anti-mouse IgG labeled with Alexa (488 nm) and SYBR Gold staining reagent were purchased from Molecular Probes (Eugene, OR). Other reagents used in this study were described previously (15).

Reporter Plasmid and Expression Constructs

The luciferase reporter construct containing the mouse CXCL9/MIG gene promoter (−328 bp, refs.14,30) and a mutant construct of the 5'-half site of the γRE motif in the CXCL9 promoter were described previously (31). The 3'-half site of the γRE motif and the κB1, κB2 and κB3 sites were mutated by site-directed mutagenesis with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutant sequences (sense strand) utilized were the following:

3'-γRE: CTCCCCGTTTgTGTctAATGGAAAGTAGAAC;
κB1: GGGAAGGAAAAGcGATTTggTAAATAAATGATCC;
κB2: CTGAGAGTAGccTTTTCgCCAGGACGATC;
κB3: GTAGAACATGCAcAAATTCgCTGGGATCTG. Lowercase letters represent the mutant nucleotides, and the underlined sequences are the consensus sequences for γRE and κB motifs. pCMV-CBP expression plasmid was kindly provided by Dr. C. K. Glass (University of California, San Diego, ref. 32). Deletion mutants of CBP were generated as a PCR fragment by the polymerase chain reaction (PCR) using pfu DNA polymerase (Stratagene, La Jolla, CA), and subcloned into pcDNA3 (Invitrogen). The deletion construct CBP 775-1779NLS contained the nuclear localization signal from SV40
T antigen at the C-terminal region of the truncated protein. Mutation in the HAT domain of CBP was generated by introducing 2 amino acid substitutions at amino acid residues 1690 (Leu to Lys) and 1691 (Cys to Leu) by using the QuickChange site-directed mutagenesis kit. This mutation was previously shown to abolish the HAT activity of CBP (33) and has been used in different systems (34,35). pRC-STAT1 was kindly provided by Dr. G. Stark (Cleveland Clinic Foundation, OH). pCMV-RelA was described previously (36).

Cell Culture and Transient Transfection

Mouse NIH3T3 cells and human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing L-glutamine, penicillin-streptomycin, and 10% FBS as described previously (5,37). Cells were transiently transfected with luciferase reporter plasmids, pRL-TK reference Renilla luciferase plasmid (Promega, Madison, WI), and expression plasmids by using Polyfect transfection reagents (Qiagen, Valencia, CA) according to the manufacturer's instruction. 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 hours, the cells were treated with IFNγ and/or TNFα for 8 hours. 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: γRE: 5'-GATCCCTTACTATAAACTCCCCGTTTATGTGAAATGGA-3'
κB1: 5'-tcgaAAAAGGGATTTCCCTAAT-3'
κB2: 5'-tcgaAGTAGGTTTTCCCCAGGA-3'
κB3: 5'-tcgaATGCAGAAATCCCTGGG-3'.
Binding reactions and antibody supershift assays were described previously (5,15).

**Immunoprecipitation and Western Blot Analysis**

Cells were washed with ice-cold PBS, harvested, resuspended in lysis buffer (50 mM Hepes [pH 7.9], 150 mM NaCl, 1 mM EDTA, 2.5 mM, 0.1% NP-40, 10% glycerol, 1mM PMSF, and 10 µg/ml of each of leupeptin, antipain, aprotinin, and pepstatin), and kept on ice for 10 min. After pre-clearing of the lysate, the whole cell lysate (~500 µg protein) was incubated with anti-V5 (1 µg) antibody or normal mouse IgG and protein G-Sepharose (50% slurry) for 16 hours at 4 C. The immunoprecipitates were washed 4 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 hr, and unbound antibody was subsequently removed by washing with PBS. 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).

**Chromatin Immunoprecipitation (ChIP)**

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-linking, the cells were resuspended in ChIP lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP-40, and 10 µg/ml of proteinase inhibitors) and sonicated with a Bioruptor sonication apparatus (Toso Electronics, Tokyo). Soluble
chromatin was collected by centrifugation, precleared with Protein G agarose, and immunoprecipitated with the desired antibodies overnight at 4 C. The immunoprecipitates were sequentially washed once with sonication buffer (50 mM Hepes [pH 7.9], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate), twice with high salt-buffer (50 mM Hepes [pH 7.9], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate), twice with low-salt buffer (20 mM Tris-Cl [pH 8.0], 250 mM LiCl, 0.5% NP-40, and 0.1% sodium deoxycholate), and twice with TE buffer before elution with elution buffer (50 mM Tris-Cl [pH 8.0], 1mM EDTA, and 1% SDS). The eluted samples were reverse cross-linked at 65 C for 5 hours and treated with RNase A and proteinase K for 1 h. The recovered DNA was purified with a DNA clean up kit (Quiagen), and samples of input DNA were also prepared in the same way. The purified DNA was subjected to PCR with a set primer and analyzed on a 2% agarose gel with SYBR Gold (Molecular Probes) staining. The stained-bands were analyzed by using a Molecular Imager (Bio-Rad). Primers for the promoter region of the CXCL9 gene were (5’ primer) TTCCACATCCAGGTAGCAACTTTG and (3’ primer) TGTTGGAGTGAGTGCCGAATTGT.

Preparation of RNA and Northern Hybridization Analysis

Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method (41). Northern hybridization analysis and cDNA probes for CXCL9/MIG and GAPDH were described previously (31,42).
Results

CBP potentiates IFNγ- and TNFα-induced CXCL9 promoter activity.

Previous studies demonstrated that IFNγ-induced STAT1 and TNFα-induced NF-κB acted synergistically in the transcription of many inflammatory genes (4,5,15,16). Since CBP has been shown to function as a transcription coactivator for various transcription factors including STAT1 and NF-κB (17-19,24-28), we wished to examine whether CBP mediated this STAT1- and NF-κB-dependent transcriptional synergy. For this purpose, we analyzed the transcriptional regulation of the CXCL9 gene, an IFNγ-inducible chemokine for activated T cells (13,14,29) and known to be cooperatively regulated by IFNγ and TNFα; although the mechanisms involved in this cooperation remain to be determined. HEK 293 cells were cotransfected 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 IFNγ and/or TNFα or left untreated before analysis of the luciferase reporter gene activity. As shown in Fig. 1, although IFNγ and TNFα alone had only a minimum effect on the CXCL9 promoter activity in the absence of CBP, costimulation with IFNγ and TNFα synergistically induced the promoter activity. When the cells were cotransfected with the expression vector encoding CBP, the cooperative response to IFNγ and TNFα was further potentiated. This result indicates that CBP functioned to mediate the synergy between IFNγ and TNFα for the transcription of the CXCL9 gene.

γ RE and κB2 sites are required for IFNγ- 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 IFNγ 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 IFNγ-responsive site that is recognized by the STAT1 tetramer (14,31), the functional
significance of several putative κB 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 κB sites and analyzed the mutant ones for their possible role in IFNγ- and TNFα-induced promoter activity. As shown in Fig. 2B, mutation of 5′-γRE or 3′-γRE abolished the cooperative response to IFNγ 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 one diminished the cooperative response to IFNγ and TNFα (lane 7). Furthermore, mutation of the κB2 site abolished the response to TNFα and markedly reduced the cooperativity for IFNγ 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 IFNγ 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 IFNγ and TNFα-stimulated cells.

An EMSA study was carried out to examine the γRE and κB DNA binding activities in nuclear extracts from IFNγ and/or TNFα-stimulated cells. As seen in Fig. 3A, IFNγ induced formation of complex I and modestly increased the binding activity of complex II (lane 2), but co-stimulation with IFNγ 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 NFκB1(p50) and RelA(p65) also reduced the binding activity of complexes II and III (lanes 9 to 11), indicating that complexes II and III contained NFκB1(p50)/RelA(p65) and NFκB1(p50) homodimer, respectively. In
this regard, NF-κB was previously reported to bind to the γRE of the CXCL9 gene as well as to GAS 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 to 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 IFNγ and TNFα, the binding activity toward these κB motifs was unchanged (Fig. 3C, lanes 4, 8, and 12). The antibody supershift assay showed that NFκB1(p50)/RelA(p65) and NFκB1(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 region 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 IFNγ/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 coimmunoprecipitation experiments (Fig. 4). HEK 293 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 IFNγ 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 (lane 5 to 8), while 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 IFNγ-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 CBP775-1779NLS 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., IFNγ-activated STAT1 preferentially interacted with the C-terminal region of CBP and TNFα-stimulated NF-κB RelA interacted with the N-terminal region of CBP.

The N-terminal and C-terminal regions of CBP are required to mediate the STAT1 and NF-κB-dependent-transcriptional 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 IFNγ/STAT1 and TNFα/NF-κB-mediated transcriptional synergy, deletion to amino acid 1779 abolished the synergistic transcription, indicating that the region between 2027 and 1779 residues was required to mediate the transcriptional synergy. This region contains the CH3 domain, 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 IFNγ/STAT1 and TNFα/NF-κB-mediated transcriptional synergy. These results demonstrate that both the N-terminal 450 amino acids and the CH3 domain of CBP are required to mediate the IFNγ/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 2058 and 2163 residues of CBP (44,45); and this region has been recently identified as the IRF-3 binding domain (IbiD, ref. 46). Interestingly, while 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 IFNγ/STAT1 and TNFα/NF-κB-mediated transcriptional synergy does not require this region.

The IFNγ/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 IFNγ/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 IFNγ and TNFα, indicating that the HAT activity of CBP is dispensable for the IFNγ/STAT1 and TNFα/NF-κB-induced transcriptional synergy of the CXCL9 promoter.
Costimulation with IFNγ and TNFα does not induce histone hyper acetylation 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 IFNγ/STAT1 and TNFα/NF-κB-induced transcriptional synergy, CBP interacts with other coactivators such as p/CAF, NcoA1(SRC-1), NcoA3(p/CIP/ACTR), which also possess HAT activity (47-49). To determine whether histone hyper acetylation 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 chromatin immunoprecipitation (ChIP) assay (Fig. 7). Initially, we monitored the acetylation status of the CXCL9 promoter in the presence of trichostatin A (TSA), an histone deacetylase (HDAC) inhibitor (50). While 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 (lane 7 to 10), indicating that the promoter region of the CXCL9 gene is deacetylated by TSA-sensitive HDAC with the cells in the quiescent state. To determine if costimulation with IFNγ and TNFα led to hyper acetylation at the CXCL9 promoter, we performed a ChIP assay with antibodies against anti-acetylated histones H3 and H4 (Fig. 7C). Treatment of cells with IFNγ or TNFα for 4 hours 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 IFNγ and TNFα-treated cells (lane 9) despite the fact that a marked synergism between IFNγ and TNFα was observed in the expression of the endogenous CXCL9 gene (Fig.7D, lane 4). Interestingly, while the level of acetylated histone H4 was also enhanced in IFNγ-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 IFNγ and TNFα-treated cells (lane 9). Thus, these results indicate that IFNγ/STAT1 and TNFα/NF-κB-induced transcriptional synergy with respect to the CXCL9 gene does not correlate with histone hyper acetylation at the promoter.

Costimulation with IFNγ 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 IFNγ and TNFα could induce a cooperative binding of STAT1 and/or NF-κB to the CXCL9 promoter (Fig. 8A). Although IFNγ alone modestly recruited STAT1 to the promoter (lane 6), costimulation with IFNγ 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 the CH3 domain (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 IFNγ/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, while IFNγ and TNFα alone had only minimum effect on the recruitment of CBP (lanes 6 and 7), costimulation with IFNγ 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 IFNγ and TNFα-treated cells. Thus, taken together these results indicate that the IFNγ/STAT1 and TNFα/NF-κB...
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 IFNγ and TNFα or bacterial LPS (2-5,15,16). We and others previously reported that IFNγ-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 IFNγ/STAT1 and TNFα/NF-κB. Our results demonstrate that simultaneous interactions of CBP with IFNγ-induced STAT1 and TNFα-activated NFκB RelA(p65) were required to mediate the transcriptional synergy. Furthermore, the IFNγ/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 IFNγ/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 γRE and the κB sites. 3) The N-terminal 450 residues and the C-terminal region between 2027 and 1779 amino acids were required for the CBP-mediated transcriptional synergy. 4) IFNγ-induced STAT1 and TNFα-activated NF-κB RelA(p65) simultaneously interacted with distinct regions of CBP. 5) Costimulation with IFNγ 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 IFNγ/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 IFNγ 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 IFNγ and TNFα induced an 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 enhansome-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 IFNγ/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 IFNγ 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 show to bind to the CH3 domain 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 IFNγ. 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 (1-450 amino acid) of CBP appeared to be required for mediating the transcriptional synergy. As was 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 (1-450) has been shown to interact with the transactivating domain of RelA (amino acids 313-550, ref. 27,28). Thus, simultaneous interaction of CBP with IFNγ-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 (IBiD) (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 IBiD, 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 IFNγ/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 IFNγ 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 IFNγ and TNFα was comparable to that with TSA treatment, an HDAC 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 p/CAF, NcoA-1(SRC-1), and NcoA-3(p/CIP/ACTR, refs. 47-49), the histone hyper acetylation 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 IFNγ 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 LPS and IL-1 induce activation of NF-κB. When the cells are exposed to stimuli that activate both signalling 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.
References


Footnotes:

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3. Abbreviation used are the following: IFN, interferon; TNF, tumor necrosis factor; CXCL, CXC ligand; IP-10, IFN inducible protein 10 kDa; STAT, signal transducers and activators of transcription; GAS, IFNγ-activated sequence; γRE, INFγ-responsive element, IRF-1, interferon regulatory factor-1; NF-κB; nuclear factor κB; MIG, monokine induced by gamma IFN; CBP, CREB-binding protein; HAT, histone acetyltransferase; CH3, cysteine-histidine-rich domain 3; HDAC, histone deacetylase; TSA, trichostatin A; ChIP, chromatin immunoprecipitation; IBiD, IRF-3 binding domain; RNA Pol II, RNA polymerase II.
Figure legends

Figure 1. Coactivator CBP potentiates IFNγ and TNFα-induced cooperative promoter activity of the CXCL9 gene.

(A) Schematic representation of a luciferase reporter construct containing the 5’-flanking sequence of the CXCL9 gene (14,30,31). The numbers above the promoter region refer to the nucleotide position relative to the transcription start site. (B) HEK293 cells were transiently co-transfected with either empty vector or the CBP expression plasmid (2µg) and the CXCL9 luciferase reporter construct (Mig-328, 1µg). Twenty-four hours after transfection, the cells were either left untreated or treated with IFNγ and/or TNFα (10 ng/ml each) for 8 hours 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 ± SEM of 3 independent experiments.

Figure 2. The γRE and κB sites in the promoter region of the CXCL9 gene are required for IFNγ 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 (2µg) and the indicated wild-type or mutant Mig-328 luciferase reporter construct (1µg). Twenty-four hours after transfection, the cells were either left untreated or treated with IFNγ and/or TNFα (10 ng/ml each) for 8 hours prior to measurement of luciferase activity. The relative luciferase activity is shown as fold induction compared with the activity of the unstimulated samples. Each column and bar represents the mean ± SEM of 3 independent experiments. (C) Individual NIH3T3 cell cultures were transiently transfected with the indicated wild-type or one of the mutant Mig-328 luciferase reporter constructs (3 µg). Luciferase activity was measured as described above. Each column and bar represents the mean ± SEM of 3 independent
experiments. (D) The nucleotides sequences of the γRE and κB sites are illustrated. Underlined sequences represent the γRE and κB motifs.

**Figure 3. γRE and κB DNA binding activities in nuclear extracts from IFNγ and TNFα-stimulated cells.**

NIH3T3 were either left untreated or treated with IFNγ and TNFα (10 ng/ml each) for 30 min before the preparation of nuclear extracts. Ten µg of each nuclear extract was analyzed for γRE (A, B) or κB (C, D) binding activity by EMSA using radiolabeled oligonucleotides as described in the Experimental Procedures. In some experiments (B, D), nuclear extracts were incubated with the indicated antibodies (1 µg) before analysis of the binding activities. Super-shifted complexes (s.s.) and non-specific binding (ns) are indicated. Similar results were obtained from 3 separate experiments.

**Figure 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; HAT, histone acetyltransferase; IBiD, IRF-3 binding domain. CBP 775-1779 contained a nuclear localization signal (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 deletions mutants were fixed and labelled with anti-V5 antibody. Immunofluorescence staining was detected by confocal laser scanning microscopy. (C, E) HEK293 cells were transiently co-transfected with V5-epitope-tagged expression plasmid together with STAT1 and RelA expression vectors.
Twenty-four hours after transfection, the cells were either left untreated or treated with IFNγ and/or TNFα (10 ng/ml each) for 1 hour. 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. Mr, relative molecular masses. (D) Whole cell extracts prepared as described above were assessed for STAT1 and RelA by Western blotting using ant-STAT1 and anti-RelA antibodies.

Figure 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 know 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). Twenty-four hours after transfection, the cells were either left untreated or treated with IFNγ and/or TNFα (10 ng/ml each) for 8 hours 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 ± SEM of 3 independent experiments.

Figure 6. CBP HAT activity is dispensable for the IFNγ/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). Twenty-four hours after transfection, the cells were either left untreated or treated with IFNγ and/or TNFα (10 ng/ml each) for 8 hours 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 ± SEM of 3 independent experiments.

Figure 7. Histone acetylation at the promoter region of the CXCL9 gene. (A) Schematic representation of the promoter region of the CXCL9 gene. The region amplified by the primer pairs used in the PCR step of the ChIP assay is illustrated. The numbers refer to the nucleotide position relative to the transcription start site. (B) Time-dependent increase in the levels of histone H4 acetylation at the CXCL9 promoter region by TSA treatment. NIH3T3 cells were treated with TSA (100 nM) for the indicated periods of time. Cross-linked chromatin fragments were prepared and immunoprecipitated with anti-acetylated histone H4. The recovered DNA was amplified by PCR with the specific primers for the promoter region of the CXCL9 gene. The amplified products were analyzed on a 2% agarose gel. DNA isolated from sonicated cross-linked chromatin fragments were used as inputs. (C) Costimulation with IFNγ and TNFα does not cooperatively induce histone hyper acetylation at the CXCL9 promoter. NIH3T3 cells were treated with IFNγ and/or TNFα (10 ng/ml each) or TSA (100 nM) for 4 hours, and cross-linked chromatin was then prepared. Soluble chromatin was immunoprecipitated with anti-acetylated histone H3 or H4. The recovered DNA was amplified and analyzed as described above. (D) Northern blots analysis of the CXCL9 mRNA expression. NIH3T3 cells were treated with IFNγ and/or TNFα (10 ng/ml each) for 4 hours as described above prior to preparation of total RNA and analysis of specific mRNA levels by Northern hybridization.

Figure 8. Enhanced recruitment of STAT1, CBP, and RNA polymerase II to the CXCL9 promoter region by costimulation with IFNγ and TNFα.
NIH3T3 cells were treated with IFNγ and/or TNFα (10 ng/ml each) for 4 hours, and cross-linked chromatin was then prepared. Soluble chromatin was immunoprecipitated with anti-STAT1 (A) or anti-CBP or anti-RNA polymerase II (B) antibody. The recovered DNA was amplified by PCR and analyzed on a 2% agarose gel as described in the legend to Fig. 7.
Fig. 1

A

Reporter: Mig-320

B

Luciferase Activity (Fold)

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Vector | CBP
Fig. 2

**A**

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**B**

**C**

**D**

sRE: AAAAGGCTTTCCGTAAAT

sRE: AATAGGTGTTCGGCAAGAA

Consensus: GGORIVYGC
Fig. 3

A

Probe: γRE

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B

Probe: γRE

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Antibody:

- S.S.
- RelA/
- NFκB1
- NFκB1/
- NFκB1

C

Probe: xB1

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D

Probe: xB2

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Fig. 4

A. Structure of CBP

Expression constructs

B. CBP 1-777 vs CBP 1758-2241

C. Transfection: CBP 1-777

IP:

IFNγ  -  -  -  -  -  -  -
TNFα  -  -  -  -  -  -  -
Blots:

α STAT1
α RelA
α V5

D. Lysates:

α STAT1  α RelA

E. IP:

IFNγ  -  -  -  -  -  -  -
TNFα  -  -  -  -  -  -  -
Blots:

α STAT1
α RelA
α V5

Transfection: CBP 775-1779  CBP 1758-2441

* : CBP 1758-2441

Mr (kDa)
Fig. 5

A

CBP Expression Vector

B

Reporter: Mig-328
Luciferase Activity (Fold)

IFNγ

TNFα

Vector

CBP

WT

1-2179

1-2027

1-1779

CBP 450-2441

Untreated

IFNγ

TNFα

IFNγ+TNFα
Fig. 6

Reporter: Mig-328

Luciferase Activity (Fold)

- Untreated
- IFNγ
- TNFα
- IFNγ+TNFα

IFNγ + +
TNFα + +

Vector
CBP
CBP mHAT
Fig. 7

A

-243 → -198
γRE xB3 xB2 xB1 TATA +57

B

INPUT
0 1 2 4 8
1 2 3 4 5

ChIP
TSA (hrs)
0 1 2 4 8
6 7 8 9 10

Acetyl H4

C

INPUT
UT IFNγ TNFα IFNγ+TNFα TSA
1 2 3 4 5

ChIP
UT IFNγ TNFα IFNγ+TNFα TSA
1 2 3 4 5

Acetyl H3
Acetyl H4

D

Northern
UT IFNγ TNFα IFNγ+TNFα
1 2 3 4

CXCL9

Tubulin
Fig. 8

A

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STAT1

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CBP

RNA Pol II
The transcriptional coactivator CBP cooperates with STAT1 and NF-κB for synergistic transcriptional activation of the CXCL9/MIG gene
Miki Hiroi and Yoshihiro Ohmori

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