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The Rel Family Member P50 Mediates Cytokine-Induced C-Reactive Protein Expression by a Novel Mechanism

Hyunjoo Cha-Molstad,* Alok Agrawal,† Dongxiao Zhang,²* David Samols,* and Irving Kushner³†

Transcription of C-reactive protein (CRP) in Hep 3B cells is induced by IL-6, acting through C/EBP isoforms and STAT3. IL-1β, which alone has no effect, greatly enhances IL-6-induced transcription by unknown mechanisms. Because IL-1β activates the NF-κB system, we explored the effects of overexpressed Rel family members on CRP expression. Unexpectedly, transactivation assays in transiently transfected Hep 3B cells showed p50 overexpression to markedly induce CRP transcription, acting in a region 3’ to −86. In the presence of overexpressed p50, IL-1β induced a 3-fold increase in CRP expression, and responses to IL-6 and to IL-6 plus IL-1β were 4-fold greater than seen in cells without p50 overexpression. In contrast, overexpressed p65 abolished CRP induction by p50 and by cytokines. EMSA studies demonstrated that recombinant p50 bound to a nonconsensus κB site overlapping the proximal C/EBP binding site on the CRP promoter. Mutation of a polypyrimidine tract in the p50-binding site inhibited the transactivating effect of cytokines. P50- but not p65-containing dimers were found in nuclei of Hep 3B cells 18 h after stimulation with IL-1β, when C/EBPβ is greatly activated, in the presence or absence of IL-6. These findings suggest that IL-1β induces nuclear translocation of p50-containing dimers and that p50 interacts with C/EBPβ activated by both IL-6 and IL-1β to induce CRP expression. The Journal of Immunology, 2000, 165: 4592–4597.

A large number of systemic and metabolic changes, collectively referred to as the acute-phase response, begin to occur within hours after inflammatory stimuli such as bacterial infection, trauma, myocardial infarction, and immunologically and crystal-induced inflammatory states (1, 2). One major change is the expression of acute-phase proteins in hepatocytes. Recent studies have focused on the mechanisms regulating expression of these major human acute-phase proteins, C-reactive protein (CRP)⁴ and serum amyloid A (SAA) in human hepatoma cell lines (3–14).

The signal transducing pathways regulating CRP induction by cytokines are not fully delineated. IL-6 induces only moderate levels of CRP gene expression in the human hepatoma cell line, Hep 3B. IL-1β alone has no effect, but when added to IL-6 causes marked synergistic enhancement of CRP gene expression (3). Transcription factors known to mediate at least part of the IL-6 effect include STAT3 and members of the C/EBP family (5, 7). C/EBP family members activate transcription in response to IL-6 through two response elements centered at −52 (7) and −219 (10), while STAT3 participates in induction of CRP through a cis-acting element centered at −108 (5). The responsive DNA sequence mediating the synergistic effect of IL-1β in the presence of IL-6 has not been identified as yet.

Many of the effects of IL-1 are mediated by the NF-κB system (15, 16). An example is SAA, which exhibits a great deal of similarity to CRP in magnitude and kinetics of induction and the cytokines required for its optimal induction in Hep 3B cells (17). IL-1 has been shown to induce SAA expression by activation of the classical NF-κB heterodimer p65/p50, with consequent physical interaction between p65 and activated C/EBPβ (11). Besides SAA, NF-κB has been shown to play a role in expression of many inflammation-associated genes including IL-6 (18), IL-1 (19), ICAM-1 (20), LPS binding protein (21), angiotensinogen (22), and G-CSF (23). Accordingly, we explored the possibility that Rel family members might similarly be involved in the IL-1β-mediated synergy observed on the CRP promoter. We found, unexpectedly, that p50 overexpression induced CRP gene expression, acting through a nonconsensus κB site overlapping the proximal C/EBP binding site on the CRP promoter, while p65 overexpression inhibited CRP gene expression, and that mutation of the p50-binding site inhibited the transactivating effect of cytokines. These findings indicate that the NF-κB system participates, in a nonclassical way, in inducing CRP gene expression in response to cytokines.

Materials and Methods

Materials

Recombinant human IL-1β and IL-6 were obtained from Biosource International (Camarillo, CA). CRP promoter deletion constructs ranging from −55 to −904 bp were generous gifts from Dr. Neal Goldman (Food and Drug Administration, Bethesda, MD). Rsv-p50 and rsv-p65 were gifts from Dr. Gary Nabel (National Institutes of Health, Bethesda, MD). PGL-2 basic plasmid, luciferase assay kits, and purified recombinant human p50 (rhp50) protein were purchased from Promega (Madison, WI). The pNF-κB-LUC vector was purchased from Clontech Laboratories (Palo Alto, CA). Abs to p50 and p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FuGENE 6 transfection reagent, DNA ligase, T4 polynucleotide kinase, and endonucleases were obtained from Roche Molecular Laboratory (Indianapolis, IN). Oligonucleotides were purchased from Life Technologies (Gaithersburg, MD) and Genosys (The Woodlands, TX). Quick-Change Mutagenesis kit was obtained from Stratagene (La Jolla, CA).

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⁴Abbreviations used in this paper: CRP, C-reactive protein; SAA, serum amyloid A; rh, recombinant human.
Cell culture

Human hepatoma Hep 3B cells were provided by Dr. G. J. Darlington (Baylor College of Medicine, Houston, TX) and were maintained in RPMI 1640 supplemented with 10% FBS. Cells were subcultured weekly after trypsinization.

Transfection and cytokine treatment

Transfection using the FuGENE 6 reagent was performed as described by the manufacturer. Cells were plated into 6-well plates in RPMI 1640 medium supplemented with 10% FBS so that the cells were 50–80% confluent (10^5–10^6) cells) on the day of transfection. Diluted transfection solution (3 μl of FuGENE 6 plus 97 μl of serum-free RPMI 1640 medium) was incubated for 5 min and mixed with 2 μg of DNA (1 μg of luciferase reporter DNA and 1 μg of expression DNA) for 15 min. The FuGENE 6/DNA mixture was then applied to Hep 3B cells for a 24-h incubation.

Plasmid construction and site-directed mutagenesis

Deletion constructs of the CRP promoter (5' flank/5' untranslated region), −55/−9, −86/−9, −125/−9, −256/−9, −339/−9, −539/−9, and −904/−9 CRP-CAT3M, were subcloned from the CAT3M vector to pGL2-basic by cleaving the promoter fragments with Xhol (5') and BgII (3') and the vector with Nhel and BgII. −157/−3 CRP-luciferase has been previously reported (3). Constructs containing a mutated C/EBP site and a p50-binding site, centered at −52 and −43 position, respectively, were generated using a Stratagene Quick-Change site-directed mutagenesis kit according to the manufacturer’s instructions. The natural sequence for C/EBP, −55GGGCCAA−40, was replaced with nucleotides, −55GAGATAC−40, using mutagenic primers (5' primer: GGAAAAATTTATTACATGGGATATT ACTTCCTACTGCTTGTTG and 3' primer: CCAAAAGCTATTGAGGATTATCCTGAAATTTTTCC). The CRP-derived putative p50 binding sequence, −45CCCT−42, was replaced with −45ATAG−42 using mutagenic primers: 5' primer, TGTGCGCCTACTACCATGTTG and complementary primer, CAAAGTCGATATTGTTGGC.

Luciferases

Luciferase reporter assays were performed following a protocol supplied by the manufacturer. Cell extracts were prepared with 50 μl of 1x reporter lysis buffer and one freeze-thaw cycle. Next, 20 μl of the cell extract was loaded onto a 96-well microtiter plate. Then, 100 μl of luciferase reagent was added, and luciferase activity was measured in a luminometer (Wallac, Gaithersburg, MD), which was programmed to read for 3 s following a 2-s measurement delay. Luciferase activity was normalized to the protein concentration of the extract measured by a Bio-Rad protein assay kit.

Cell culture

Confluent Hep 3B cells were washed twice and exposed to 10 ng (100 U)/ml of IL-6, 20 ng (200 U)/ml IL-1β, or the combination of the two cytokines for 24 h.

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Results

Overexpression of the NF-kB subunit p50 transactivates CRP gene expression, while p65 inhibits it

Overexpression of p50 in Hep 3B cells increased basal expression of a −157/−3 CRP-LUC construct 10-fold (compare lanes 1 and 5, Fig. 1). Similar patterns of response were seen in other CRP-LUC deletion constructs studied, −904/−9, −539/−9, −339/−9, and −256/−9 (not shown). The effects of cytokine treatment on p50-transfected cells were substantially greater than and differed somewhat from those seen in cells not transfected with p50 DNA. IL-1β caused a 3-fold increase in luciferase expression in cells overexpressing p50 but had no effect in cells not transfected with the p50 DNA. For cytokine treatment, the transfected cells were washed with serum-free medium twice and exposed to 10 ng (100 U)/ml of IL-6, 20 ng (200 U)/ml IL-1β, or the combination of the two cytokines for 24 h.

Nuclear extract preparation

Hep 3B cells were washed and incubated in serum-free medium for 16 h and then left untreated or treated either with 20 ng (200 U)/ml of IL-1β or with the combination of 10 ng (100 U)/ml of IL-6 and 20 ng (200 U)/ml of IL-1β for 15 min or 18 h. The nuclear extracts were prepared according to Schreiber et al. (24) with some modifications. Cells (70–80% confluent) were washed with cold PBS and resuspended in 3× packed cell volume of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 0.1 mM sodium orthovanadate, and 0.2% Nonidet P-40) by gentle pipeting on ice for 15 min. The nuclei were pelleted in a microcentrifuge at 12,000 × g for 30 s and resuspended in 3× packed nuclear volume of high salt buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM PMSF). The suspension was rocked gently for 30 min at 4°C followed by microcentrifugation at 12,000 × g for 10 min at 4°C. The protein concentration in supernatant/nuclear extracts was determined with the BioRad protein assay kit.

EMSA

The oligonucleotide containing the C/EBP-binding site derived from the CRP promoter and the NF-κB consensus oligonucleotide used in the EMSAs were: 5'-TACATAGTGGCCGAACATTCCCTT-3' and 5'-GATCCAAAGGGGATTTCCAGT-3', respectively. A κB consensus oligo that preferentially binds p50 homodimers was designed according to a published sequence (25). Complementary oligonucleotides were annealed and end-labeled with [γ-32P]-ATP using T4 polynucleotide kinase; EMSA was carried out as published previously with some modification (5). Recombinant p50 (38 ng/μl gel shift units) or nuclear extracts (6 μg protein/μl extract) were incubated with about 0.5 ng of labeled double-stranded oligonucleotide probe (sp. act., ~5×10^6 cpm/ng) in gel shift incubation buffer (16 mM HEPES, pH 7.9, 40 mM KCl, 1 mM EDTA, 2.5 mM DTT, 8% Ficoll, and 1 μg of poly(dI-dC)) for 20 min at room temperature. In supershift experiments, Ab (2 μg) was added to the reaction mixture and incubated on ice for 15 min before addition of the probe. In competition experiments, a 200-fold molar excess of unlabeled oligo was added to the binding reactions and incubated on ice for 15 min before addition of the probe. The DNA-protein complexes were resolved by electrophoresis on a 5% native polyacrylamide gel in 0.25 TBE (1 TBE: 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA) at 10 V/cm. The gels were dried and analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA) in a phosphor imager.
combined both the C/EBP-binding site and this nonconsensus 22-bp probe (lane 2), derived from the CRP promoter, that formed an EMSA using purified rhp50 as a protein source and a To test the hypothesis that p50 might bind to this site, we per-
formed a mutational analysis to more precisely define the p50-binding site on this short 22-bp segment of the CRP promoter. We determined luciferase activity in Hep 3B cells cotransfected with either of these plasmids and a construct containing five κB sites linked to a minimal promoter and a luciferase reporter (pNF-κB-LUC). As expected, cells overexpressing p65 exhibited a marked transactivation activity on pNF-κB-LUC expression, while cells overexpressing p50 did not stimulate this construct and cotransfection with p50 diminished the effect of p65 (Fig. 2).

The p50 response region lies 3′ to −86

To map the position of the p50-responsive sequence element, we determined the effect of p50 overexpression on a series of eight CRP promoter deletion constructs varying from 904 to 55 bp 5′ flanking DNA (Fig. 3). P50 overexpression in the absence of cytokines induced a 6- to 10-fold increase in expression of the luciferase reporter compared to untreated controls in all constructs but −55. The data in Figs. 1 and 3 are consistent with the major p50 response region lying 3′ to nucleotide −86. Accordingly, we examined the sequence of this region for a possible p50-binding site and noted that the C/EBP-binding site centered at −52, previously shown to be critical to CRP induction (6, 8), overlaps a nonconsensus κB site (−560AAAATCCCTT −411) (Fig. 4).

P50 binds to a nonconsensus κB site derived from the CRP promoter overlapping a C/EBP-binding site

To test the hypothesis that p50 might bind to this site, we performed an EMSA using purified rhp50 as a protein source and a 22-bp probe (−41/ −63), derived from the CRP promoter, that contained both the C/EBP-binding site and this nonconsensus κB site (Fig. 5). We found that rhp50 bound to this probe (lane 1). The resulting band was supershifted by an Ab to p50 (lane 2). The specificity of this interaction was shown by competition with an excess of either self-oligo or consensus κB site oligo (lanes 3 and 10), while a consensus C/EBP-binding site oligo did not compete (lane 11).

In contrast, binding of rhp50 to the probe was not competed by oligos with mutations in a polypyrimidine tract (lanes 6−8), indicating that binding of p50 to this nonconsensus κB site requires an intact polypyrimidine sequence. Disrupting two of the three purine residues that overlapped the core C/EBP site and nonconsensus κB site (M6) affected binding of p50 to the wild-type oligo only moderately (lane 9). Similar results were obtained when these oligos were used as labeled probes.

Mutation at the nonconsensus κB sequence overlapping the C/EBP-binding site inhibited the inducing effect of cytokines

We next explored the roles of the nonconsensus κB sequence and of the adjacent C/EBP site in induction of CRP by cytokines. We performed transient transactivation assays using −125/+3 CRP-LUC constructs containing mutations at either the polypyrimidine p50-binding site at −43 or the C/EBP site at −52. In cells transfected with constructs bearing the C/EBP mutation (Fig. 6), basal transactivation was greatly diminished with deletion below −86 in the absence of cytokine. Hep 3B cells were transiently cotransfected with CRP promoter deletion constructs and an expression vector for p50 as indicated. Transcription was measured as luciferase activity, and the activity of each reporter plasmid alone was set to 1. The bars indicate the SD from at least three independent experiments.
expression was virtually abolished, as were both the modest response to IL-6 and the robust response to IL-6 plus IL-1β. The effects of the p50 site mutation were somewhat less marked. Basal expression was halved, the IL-6 response was abolished, and the response to IL-6 plus IL-1β decreased by about half compared to cells transfected with wild-type constructs. No effect of IL-1β alone was observed in any condition. The 8.8-fold incremental effect induced by IL-1β over that observed with IL-6 alone in wild-type cells did not differ substantively from the 8.6-fold increase observed in cells transfected with constructs bearing the p50 site mutation. The finding that mutation both at the C/EBP-binding site and at the adjacent nonconsensus kB sequence inhibited the inducing effect of cytokines demonstrates that binding of both C/EBP proteins and p50 to DNA is required to achieve optimal transactivation in response to cytokines. 

P50, but not p65, is present in nuclei of unstimulated cells and of cells 18 h after stimulation with IL-1β

We determined whether p50 and p65 are activated in Hep 3B cells 18 h after cytokine stimulation, a time of marked C/EBP activation. We performed EMSA using a consensus kB probe to ensure that any p65 present in the extracts could be detected. Nuclear extracts from untreated Hep 3B cells formed a complex with the probe, which was supershifted by an Ab to p50 but was not supershifted by an Ab to p65 (Fig. 7). Stimulation with IL-1β for 15 min yielded a band supershifted by Abs to both p50 and p65. However, when IL-1β stimulation was continued for 18 h, the resulting band was supershifted by an Ab to p50, but only minimally altered by an Ab to p65. Comparable results were seen following stimulation with IL-6 plus IL-1β, while IL-6 alone did not yield results different from unstimulated cells (data not shown). These findings indicate that p50- but not p65-containing dimers are present in nuclei of Hep 3B cells, both unstimulated and 18 h after stimulation with IL-1β.

Discussion

Our major findings were: 1) overexpressed p50 induced expression of a CRP-luciferase construct, acting through a nonconsensus kB sequence overlapping the proximal C/EBP site of the CRP promoter, while overexpressed p65 repressed both basal and cytokine-induced CRP expression; 2) binding of p50 to this site was dependent on an intact pyrimidine-rich sequence; 3) complete transcriptional activation of the proximal CRP promoter by IL-6 and IL-6 plus IL-1β required that both the proximal C/EBP-binding site and this nonconsensus kB sequence be intact; and 4) p50- but not p65-containing dimers were found in nuclei of Hep 3B cells, 18 h after stimulation with IL-1β. We conclude that IL-6 plus IL-1β causes activation of C/EBP species and p50-containing dimers, with consequent binding of these transcription factors to adjacent sites on the CRP promoter. Furthermore, because this effect is inhibited by overexpressed p65, we conclude that p50 homodimers (or alternatively the heterodimers p50/c-rel or p50/rel B), but not the p50/p65 heterodimer, are acting as activators of CRP gene expression.

Transactivation by overexpressed p50 alone is unusual because p50 lacks a transactivation domain. In general, it is p65, and not p50, that is the positive effector of inflammation-associated genes (9, 11, 12, 18, 23, 26–28). There are only rare studies in which overexpressed p65 was found to act as a repressor (29). Interestingly, SAA, which accumulates in blood with kinetics similar to those of CRP following an acute-phase stimulus and whose induction in Hep 3B cells also requires the combination of IL-6 and IL-1 (11), uses p65 as an activator of its transcription in contrast to CRP (30). Our observation that p65 overexpression inhibits the inducing
effects of both p50 and of cytokines can be explained by either displacement or sequestration of p50, rendering it unable to participate in transactivation. The latter is more likely because p65-containing dimers are not detected by EMSA when the nonconsensus κB site in the CRP promoter is used as a probe and nuclear extracts known to contain p65 (Fig. 5) are employed (A. Agrawal et al., manuscript in preparation).

The functional importance of p50 binding to the CRP promoter was substantial. While mutation in the proximal C/EBP-binding site completely inhibited the effects of IL-6 and IL-6 plus IL-1β, consistent with previous reports demonstrating the importance of this binding site (6, 10), mutation of the putative p50-binding site similarly completely inhibited IL-6 activity and greatly reduced the effect of IL-6 plus IL-1β. This finding indicates that p50 plays an active role in response to IL-6, even though p50 is not known to be activated by IL-6 and EMSA studies did not show IL-6 to induce nuclear p50 in our system.

The mechanism by which p50 transactivates CRP is uncertain. Several recent reports (31–33) have demonstrated that p50 or p52 homodimers can act as transcriptional activators in systems in which the IκB family member Bcl-3 acts as a coactivator. We have not found Bcl-3 to be present in p50-DNA complexes in our system. This finding suggests that this mechanism is not responsible for the inducing effect of p50 on CRP, although our findings are not definitive. Indeed, our finding that mutation of the C/EBP-binding site abolishes the response to p50 (A. Agrawal et al., manuscript in preparation) supports our view that an alternative mechanism, dependent upon the transactivating domain of C/EBP proteins, is responsible.

The presence of p50-containing dimers in nuclei of unstimulated cells of various cell types is well documented (34, 35), and indeed we have shown such species in our system (Fig. 7). The finding of p50- but not p65-containing dimers in nuclei of Hep 3B cells 18 h after stimulation with IL-1β is consistent with interaction between C/EBPβ, known to be greatly activated at this time point, and p50. Physical interaction in vitro between p50 and C/EBPβ has been reported (36), but a functional role for such binding has not previously been demonstrated. In addition, physical and functional interaction between Rel domains of p65 and the bZIP domain of C/EBP species has also been observed (37). Our results are consistent with a model in which p50 functionally interacts with cytokine-activated C/EBP species to activate transcription. It is not as yet clear whether physical binding of p50 to C/EBP species occurs in this system.

We propose a model in which p50 enhances cytokine-induced transcription of CRP indirectly by affecting functional activity of the transcription factor C/EBPβ, which can be activated by both IL-6 and IL-1β (Fig. 8). We speculate that p50 may physically interact with C/EBP, enhancing its DNA binding affinity or stabilizing C/EBP binding to DNA. Alternatively, p50 binding to the nonconsensus κB site may cause a conformational change in DNA, facilitating C/EBP binding to DNA or bringing C/EBP in closer contact with the basal transcription machinery.

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**FIGURE 7.** P50-containing dimers are present in the nuclei of unstimulated Hep 3B cells and 18 h after IL-1β stimulation. EMSA was performed using nuclear extracts from untreated and cytokine-treated Hep 3B cells for the indicated periods of time using a NF-κB consensus oligo as a radiolabeled probe. Unlabeled self competitor (lanes 2, 7, 12, 17, and 22) was used in 200-fold excess. Abs recognizing p50 and p65 and normal rabbit IgG were added to the nuclear extracts before the addition of the labeled probe. DNA-protein complexes were separated on a 5% native polyacrylamide gel, and the results were analyzed by phosphorimager. Detailed methodology is given in Materials and Methods. A representative EMSA is shown.

**FIGURE 8.** A model for cytokine-induced transcription of CRP. IL-6 activates C/EBPβ with consequent nuclear localization after 18 h. IL-1β leads to nuclear localization of p50 homodimers, which bind to the nonconsensus κB site, facilitating and stabilizing the binding of C/EBP to its cognate site, perhaps by inducing a conformational change in DNA (step 1). P50 may also physically interact with C/EBP, bringing it in closer contact with the basal transcription machinery (step 2). P50 may then remain bound to DNA or may detach once it has facilitated C/EBPβ binding.
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References


