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β-Catenin binds to the downstream region and regulates the expression C-reactive protein gene

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ABSTRACT

C-Reactive protein (CRP) is a major acute-phase response protein, which is activated by various cytokines. We investigated the mechanism of TNF-α-induced CRP expression and found that the p50 subunit of NF-κB was responsible for the transcriptional activation of CRP. Since the p50 protein acts as a positive regulator of CRP expression without an inherent transactivation domain, we looked for an interaction partner that could provide p50 with such a domain. We found that β-catenin enhanced the expression of a CRP mRNA in concert with p50 subunit. Protein–protein interaction between p50 and β-catenin was important for CRP expression and their interactions to CRP promoter were induced after TNF-a treatment. Since gene expression depends upon the proximity of promoters and distal regulatory sites, we explored the long-range genomic interaction at the CRP locus by chromosome conformation capture (3C). We identified a binding site for β-catenin in the downstream of CRP gene by 3C and confirmed TNF-α-induced association of β-catenin and p50 by chromatin immunoprecipitation and co-immunoprecipitation assays. Our findings provide evidence that transcription of the CRP gene depends upon p50 and β-catenin proteins, which is accompanied by close proximity between promoter and the downstream region of CRP gene.

INTRODUCTION

NF-κB/Rel is a critical transcription factor controlling innate immunity, inflammation, cell survival and tumorigenesis (1–3). There are five structurally related members of the mammalian NF-κB/Rel family of proteins: p50, p52, p65/Rel A, Rel B and c-Rel. Genetic analysis has revealed the functional specificities of these NF-κB subunits and shown that the formation of dimers of NF-κB subunits and the exchange of subunits are important for modulating NF-κB activity (4). To understand the functional specificity of NF-κB target genes, it is necessary to identify protein–protein as well as DNA–protein interactions involving the NF-κB subunits and its target DNA elements.

Although the p65 subunit of NF-κB is the major transcriptional activator of many of NF-κB target genes, other subunits are likely to play important roles in particular NF-κB-mediated processes. For example, the p50 and p52 subunits of NF-κB appear to be important in inflammation and apoptosis (5,6). In some cases, p50 by itself without a bound p65 subunit, actually activates transcription of target genes. Because it is unusual for proteins without associated transactivation domains to activate transcription, it is likely that transcriptional co-activators are involved in this p50-mediated transcription (7). Hence identification of transcriptional co-activators of the p50 subunit should throw light on its target gene specificity.

Several natural promoters have been reported to be activated by the p50 subunit but not by the p65 subunit. We chose the C-reactive protein (CRP) promoter as a model for p50-dependent transcription (8). CRP is a major human acute-phase protein whose rate of synthesis can increase in inflammatory states (9). There is increasing evidence that it is not merely an important risk marker but also has a role in the pathogenesis of atherosclerosis (10). It is induced by various cytokines, such as interleukin-6 (IL-6), interleukin-1 (IL-1) and TNF-α (11). It was previously proposed that IL-6 stimulates CRP expression, and one of the non-consensus κB sites overlapping the C/EBP-binding site was identified as a p50-responsive element (12). Even though it has been proposed that NF-κB p50 subunit plays important roles in CRP expression, the transcriptional activator for p50 protein has not been identified in the cases of these inflammatory signals.

β-Catenin is a multifunctional protein that plays critical roles in cell adhesion as well as Wnt-activated tumorigenesis (13). When β-catenin levels rise, it accumulates in the nucleus, where it interacts with DNA-bound TCF/LEF

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family proteins to activate the transcription of various target genes. β-Catenin binds to diverse proteins responsible for specific transcriptional regulation and chromatin remodeling in the nucleus; however, the nuclear protein complexes involving β-catenin remain to be identified. It is especially important to decipher protein–protein interaction on the specific target genes of β-catenin.

We searched for a transcriptional co-activator that might provide p50 with a transactivation domain at the CRP promoter and found that β-catenin interacts with the p50 subunit. We also used the technique of Chromatin Conformation Capture (3C) (14,15) to identify possible interaction sites forming chromatin loops to the CRP promoter. Interestingly, we identified binding sites for TCF/LEF which binds to β-catenin, not in the standard upstream region but in the downstream of the CRP gene (16). Most significantly, we also showed that the interaction of the p50 on the CRP promoter and the β-catenin on CRP downstream was involved in CRP activation upon TNF-α treatment. Our findings provide interesting evidence that transcription of the CRP gene depends upon the interaction between p50 and β-catenin and direct long distance interactions of the bound DNA.

MATERIALS AND METHODS

Cell culture and transfection

Cells of 293T were cultured in DMEM containing 10% fetal bovine serum (BRL Life Technology, Inc.). HepG2 cells were cultured in MEM containing 10% fetal bovine serum. Transfection of cells was performed using lipofectAMINE transfection reagent (Invitrogen). pCMV-β (50 ng) was co-transfected in each case to normalize transfection efficiency. After 24 h of incubation, cells were cultured under serum-free conditions for 2 h followed by treatment with 30 ng/ml TNF-α for indicated times. pSUPER-β-catenin (1.5 μg) or pSUPER-p50 was transiently transfected for knock down of p50 and β-catenin protein. After 72 h of transfection, cells were cultured under serum-free conditions for 2 h followed by treatment with 30 ng/ml TNF-α for 8 h before harvest.

Reagents and antisera

Recombinant human TNF-α, phosphatase inhibitor cocktail and protease inhibitor cocktail were purchased from Sigma-Aldrich. Restriction and modifying enzymes were from Roche Molecular Biochemicals. Anti-p50 mouse monoclonal antibody (sc-8414) and Anti-TCF-4 rabbit polyclonal antibody (sc-13027) were purchased from Santa Cruz Biotechnology. Anti-FLAG M2 mouse monoclonal antibody (F3165) was from Sigma-Aldrich. Anti-β-catenin mouse monoclonal antibody (610154) was from BD Transduction Laboratories. Anti-TCF-1 rabbit polyclonal antibody was generated by KOMA Biotechnology.

Plasmids

Human p50 expression clones were kindly provided by Dr Young Mee Kim (Asan Medical Center). Human p65/RelA expression clone were previously reported (17). Stable β-catenin (S37A) was previously reported (18). FLAG-p50 plasmid was composed of human p50 cDNA (nucleotides 52–1200 of Genbank/Bank Accession NM_003998) in the pCMV-tag2 mammalian expression vector (Stratagene, La Jolla, CA, USA). BAC (bacterial artificial chromosome) clones were purchased from ResGen (Catalog no: RPCI-11.C. Clone ID: RP11-806E24). To make pSUPER-β-catenin and pSUPER-p50, specific oligonucleotides (listed in Table 1) were synthesized (Bioneer) as previously reported (19,20). To anneal the oligonucleotides, the mixture was incubated at 95°C for 5 min, and was cooled slowly. This mixture was ligated into pSUPER vector (Oligoengine) that had been digested with BglII and HindIII.

Immunoprecipitation and western blot analysis

Whole-cell extracts were centrifuged at 12 000 r.p.m. for 15 min to obtain clear lysates and incubated either with anti-FLAG antibody or with normal rabbit serum for 2 h, followed by the incubation with protein G-Agarose beads (BRL Life Technologies) for 3 h and precipitation. Western blot analysis was previously described (21).

Chromatin immunoprecipitation (ChIP)

Cells of 293T were cultured under serum-free conditions for 2 h followed by the treatment with 30 ng/ml TNF-α for 1 h. Cells were fixed in 1% formaldehyde at room temperature and stopped the cross-liking reaction in 125 mM glycine. Cells were lysed with 200 μl lysis buffer [20 mM HEPES (pH 7.9), 10 mM NaCl, 1 mM DTT, 1 mM PMSF, 10 μl protease inhibitor cocktail] for 15 min at 4°C. Nonidet P-40 (5 μl of 0.5%) was added and centrifuged. The pellets were washed with lysis buffer and resuspended in nuclear lysis buffer [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 300 μM phenylmethylsulfonyl fluroride, 10% glycerol, 1% Triton X-100, phosphatase inhibitor cocktail and protease inhibitor cocktail] and incubated for 30 min at 4°C. Resuspended cells were sonicated (BRASON sonifier 250, duty cycle 60%, power control 100% 12 sec 2X), centrifuged for 10 min and the supernatants were collected. The chromatin fragments were then cleared with 1 μg of preimmune serum and protein G-Sepharose (30 μl of 50% slurry in 1 × PBS) for 2 h at 4°C. Immunoprecipitation was performed for 12–24 h with anti-p50, anti-β-catenin or anti-Tcf-4 antibody at 4°C. Anti-GST or normal rabbit IgG was used as a control. Following immunoprecipitation, 30 μl of protein G-Sepharose was added and incubated for 3 h. Sepharose beads were collected and washed sequentially for 3 min in low salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl] and LiCl wash buffer [0.25 M LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)] three times. Beads were washed three times with TE buffer and extracted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Elutes were heated at 65°C for 6 h to reverse the formaldehyde cross-linking.

Table 1. Primers

	Sequence
shRNA primer	
pSUPER-β-catenin	5′GATCCCGTGGGTGGTATAGAGGCTC TTCAAGAGAGAGCCTCTATACCACC CACTTTTTGGAAA-3′
	5′AGCTTTTCCAAAAAGTGGGTGGTAT AGAGGCTCTCTCTTGAAGAGCCTCT ATACCACCCACGG-3′
pSUPER-p5O	5′GATCCCTATTAGAGCAACCTAAACA TTCAAGAGATGTTTAGGTTGCTCT AATATTTTTGGAAA-3′
	5′AGCTTTTCCAAAAATATTAGAGCA ACCTAAACATCTCTTGAATGTTTA GGTTGCTCTAATAGG-3′
RT-PCR primer	
Forward	5′-CCTATGTATCCCTCAAAGCA-3′
Reverse	5′-CCCACAGTGTATCCCTTCTT-3′
Luciferase primer	
12-1 F	5′-TACTCTCATTCTATCATTATTCCAA-3′
12-2 F	3′-ATAACCTCTTTACGCCTCAGT-3′
12-2 R	5′-CTTCTATGCTTACAAATGGTTATC-3′
3C-CRP primers	
Upstream anchor	5′-CTGTCCCACCACTCTCTATCTGA-3′
Fragment 1	5′-GACACATGAATAGTGGCTTTGCTC-3′
Fragment 2	5′-CATCGCAAAGAGGCTAAACTC-3′
Fragment 3	5′-GGGATATAATCTCAATCTCCTGGTG-3′
Fragment 4	5′-AGCAAAAGAGCAAAGGGAGA-3′
Fragment 5	5′-CTGATGCCTACTATAATGGATG TATTTC-3′
Fragment 6	5′-ACCCAGTCCAGATTAGGTCAGG-3′
Fragment 7	5′-GGCTATCAACATTCATCTCCTCAT-3′
Downstream anchor	
Fragment 8	5′-CTTAAATTCTATACGTAAGTGAGGGG AT-3′
Fragment 9	5′-CGGTCCAAAAGAATCAGAATTT GA-3′
Fragment 10	5′-GTGGAGTCTCTGAATCAGCCTTG-3′
Fragment 11	5′-GATTGACAAACAACAGAGCCTTG AC-3′
Fragment 12	5′-CTGGTCTCTAAACATGGAGTTTT CC-3′
Fragment 13	5′-AGATCCCCCATGAGTGATTTAG TG-3′
Fragment 14	5′-CCCTCTTCACTAGGTATGAAGCA AG-3′
Fragment 15	5′-CACTATCTGAGGTCATCTGAGG AG-3′
Fragment 16	5′-GCTTCTGGGAATAAAGCAGGC-3′
Fragment 17	5′-CAACAGGGAAATCCTCGAATATG-3′
3C-GAPDH primers	
GAPDH 1	5′-GTTTCCATAGGACCTGCTGCG-3′
GAPDH 2	5′-GTTTTACACTGG GCACTTGAG GTC-3′
ChIP-CRP primers	
Fragment 4 Forward	5′-TCTCCCTTTGCTCTTTTGCT-3′
Fragment 4 Reverse	5′-CAATAAGTTCCAATGAAATA GACA-3′
Fragment 12 Forward	5′-ATAACCTCTTTACGCCTCAGT-3′
Fragment 12 Reverse	5′-CTTCTATGCTTACAAATGGTTATC-3′
Fragment 13 Forward	5′-GGCTGATGGACTTAGGTAATACT-3′
Fragment 13 Reverse	5′-TGACCAAACTGAGCGAACTC-3′

DNA fragments were purified by phenol extraction and ethanol precipitation. Purified DNA fragments were amplified with PCR. Primers used in this assay are listed in Table 1.

Chromosome conformation capture (3C)

Cells of 293T were cultured under serum-free conditions for 2 h followed by treatment with 30 ng/ml TNF-α for 1 h. Cells of 1×10^7 were cross-linked with 2% formaldehyde in 1× PBS (4.5 ml) at room temperature for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. Cells were washed with 1× PBS, resuspended in 1 ml cell lysis buffer [10 mM Tris (pH 8.0), 10 mM NaCl, 0.2% NP-40 and protease inhibitors], and incubated on ice for 10 min. Samples were kept on ice from this point forward. Cell lysis was completed with ten strokes of a Dounce homogenizer (pestle A). Nuclei were washed with 0.5 ml of the 1× BglII restriction enzyme buffer and resuspended in 762 μl of BglII restriction enzyme buffer. SDS was added to a final concentration of 0.1% and incubated for 15–60 min at 37°C while shaking. Triton X-100 was then added to the final concentration of 1%. Digestion was performed with 800 U of BglII at 37°C overnight. The reaction was stopped by adding SDS to a final concentration of 2% and incubated at 65°C for 30 min. The samples (50 μl) were diluted into a 950 μl ligation reaction buffer (5 ng/μl) containing 1% Triton X-100, 1× ligase buffer (NEB) and incubated for 1 h at 37°C. Ligation was started by adding 4000 U of T4 DNA Ligase (NEB) and incubated for 4 h at 16°C. Reactions were stopped by adding EDTA to a concentration of 10 mM. Samples were treated with Proteinase K (100 μg) at 65°C to reverse cross-links, incubated with RNase A (0.5 μg) for 30 min at 37°C, extracted with phenol:chloroform:IAA 2 times, chloroform 2 times, and ethanol precipitated with glycogen. Samples were redissolved in deionized water. Genomic sequences of CRP locus (chr1:156475000-156525000) and their BglII restriction enzyme sites are obtained from UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). PCR primers are designed by PREMIER biosoft program (http://www.premierbiosoft.com/netprımer/Netprlaunch/netprlaunch.html) and UCSC In-Silico PCR program (http://genome.ucsc.edu/cgi-bin/hgPcr?command = start). Primers are designed only for non-repetitive sequences. To normalize the differences of the primer efficiency, BAC control template was prepared by digesting with BglII followed by the random ligation. PCR reaction was performed with the control template in parallel with 3C templates. To equalize for the differences in template amount or quality, PCR at the CRP locus were normalized to a control interaction at the GAPDH locus. Primers used in this assay are listed in Table 1.

Cloning of −125 CRP Luc-2 and −125 CRP Luc-1-2 construct cloning

-125 CRP luciferase reporter was kindly provided by Dr Irving Kushner (Case Western Reserve University). To generate the chimeric reporter containing the downstream elements of CRP gene, the genomic fragments were cloned on the downstream of luciferase gene. Genomic DNA was prepared from 293T cells and two genomic fragments are amplified with two primer sets (12-1F, 12-2R and 12-2 F, 12-2R in Table 1), which contain one or two TCF/LEF-binding sites. PCR products were cloned in a

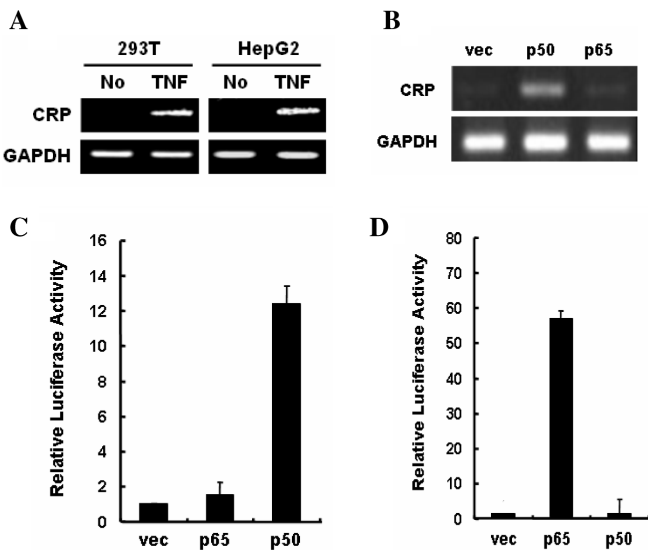

Figure 1. The NF-κB p50 subunit induces CRP transcription. (**A**) RT-PCR analysis of CRP mRNA. Cells of 293T and HepG2 were incubated with or without TNF-α for 6 h after serum starvation. GAPDH was used as a control. (**B**) RT-PCR analysis of CRP mRNA after transfection of p50 or p65 subunit in 293T cells. (**C**) Luciferase assay of the -125 CRP promoter in 293T cells. Cells were transfected with the -125 CRP Luc reporter and co-transfected with either NF-κB p50 or p65 expression vectors. After 24 h luciferase activity was determined and normalized with β-gal activity. It is shown as relative luciferase activity. Results are the average of three independent experiments. (**D**) Luciferase assay with the consensus 3X-κB Luc reporter.

pCR2.1-TOPO vector (Invitrogen) and digested with BamHI and SalI to clone into -125 CRP Luc reporter. -125 CRP Luc-2 was constructed by cloning a BamHI/Sal fragment having PCR product with 12-2 F and 12-2 R primers, which contains second putative TCF/LEF-binding site within fragment 12. -125 CRP Luc-1-2 was constructed by cloning a BamHI/Sal fragment having PCR product by 12-1 F and 12-2 R primers which contains first and second putative TCF/LEF-binding site within fragment 12. After cloning, -125 CRP Luc reporters were cut by BamHI and used in luciferase assay in some cases.

Reverse transcription-PCR analysis

RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed. Samples were amplified at 95°C for 30 s, 51°C for 30 s, 72°C for 30 s for 30 cycles. The primers for CRP were as follows. F, 5′-CCTATGTATCCCTCAAAGCA-3′ R, 5′-CCCACAG TGTATCCCTTCTT-3′.

RESULTS

NF-κB p50 and β-catenin are required for CRP gene transcription

Since CRP expression is stimulated by various cytokines, we examined how TNF-α induces CRP expression. RT-PCR analysis showed that TNF-α stimulated the transcription of the CRP gene in both 293T and HepG2 cells (Figure 1A). When we used the -125 CRP Luc reporter that contains the CRP promoter, luciferase activity was also enhanced after TNF-α treatment in two cell lines (data not shown). Since NF-κB is a key mediator of the TNF-α-induced expression of many genes, and the CRP promoter contains a non-consensus κB-binding site (AAACTCCCTTA) on -50 to -40, we tested the effect of overexpressing either the p50 or the p65 subunit of NF-κB on CRP mRNA expression. As shown by RT-PCR analysis (Figure 1B) and luciferase assays with the -125 CRP promoter (Figure 1C), overexpression of p50 increased CRP promoter activity in the 293T cell line. On the other hand p65 overexpression did not stimulate the promoter activity. To confirm that the p50 and p65 plasmids possessed the expected biological activity, we determined the luciferase activity transcribed from consensus κB sites (NF-κB Luc) in 293T cells. As expected, overexpression of p65 markedly activated the NF-κB-Luc reporter whereas overexpression of p50 did not (Figure 1D). This suggests that the TNF-α-induced CRP transcription may be dependent on the p50 subunit of NF-κB acting on the non-consensus κB site in the -125 CRP promoter.

Because the p50 subunit of NF-κB lacks a transactivation domain, transcriptional activation by p50 alone is unusual (7,22). We searched for a putative co-activator that could provide p50 with a transactivation domain during TNF-α signaling. Since TNF-α activates NF-κB signaling and PI3-Kinase/Akt pathway by which β-catenin nuclear translocation is activated, we chose β-catenin as a putative binding partner for NF-κB p50 protein. In addition, because several reports suggested that β-catenin and NF-κB may physically interact in basal level (23,24), we reasoned that β-catenin could provide the transactivation domain to p50 subunit. We also showed by RT-PCR analysis that β-catenin and p50 acted in concert to enhance the transcription of the endogenous CRP gene in the 293T and HepG2 cell lines (Figure 2A). These results suggest that β-catenin contributes to the transcriptional activation of NF-κB p50 on the CRP promoter, and may thus act as a transcriptional co-activator of the p50 subunit.

To establish the critical roles of the NF-κB p50 subunit and β-catenin in CRP gene transcription, we knocked down the expression of the two proteins using pSUPER-shRNAs in HepG2 cells. To confirm the efficacy of the shRNAs, we performed western blot analyses with anti-p50 and anti-β-catenin antibodies (Figure 2B). The shRNA for p50 greatly reduced the steady-state level of the p50 precursor protein, p105, and lowered somewhat the level of p50 protein. The shRNA for β-catenin caused significant reduction of the β-catenin protein level. We next tested whether the shRNAs inhibited TNF-α-induced CRP gene expression (Figure 2C). Knockdown of p50 resulted in a substantial reduction of CRP mRNA, which can be explained by the major roles of the NF-κB p50 in basal and activated CRP transcription. In addition, reduction of CRP mRNA was also observed when shRNA for β-catenin was introduced. These results suggest that NF-κB p50 and β-catenin are important components of TNF-α-induced CRP gene expression.

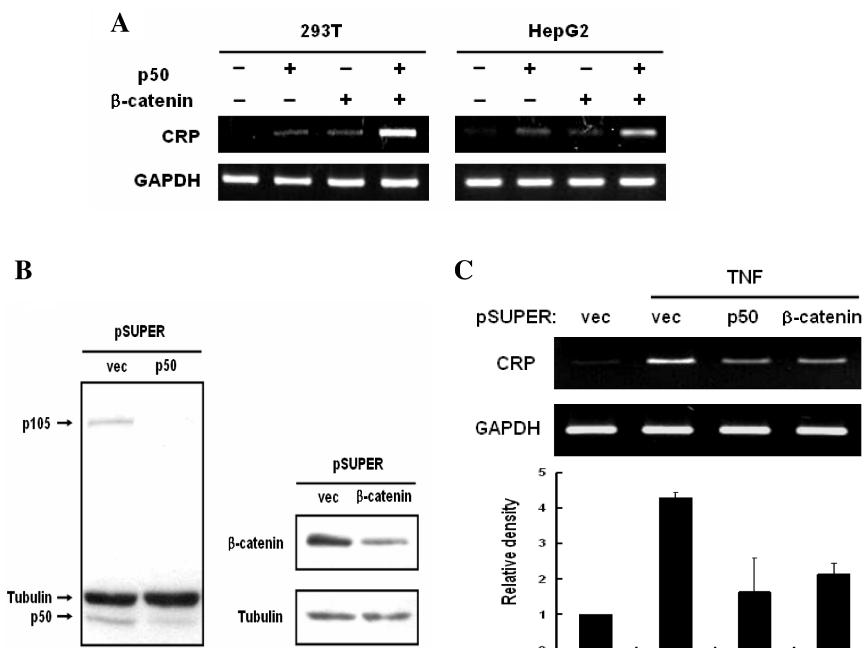

Figure 2. NF-κB p50 and β-catenin are indispensable for TNF-α-induced CRP expression. (**A**) RT-PCR analysis. Cells were transfected with either NF-κB p50 and/or stable β-catenin expression vectors. (**B**) Western blot analysis after shRNA transfection. Cells were transfected with either pSUPER vector (vec), pSUPER-p50 shRNA (p50) or pSUPER-β-catenin shRNA (β-catenin). Expression of p105 and p50 was analyzed with anti-p50 antibody. Anti-β-catenin antibody was used to test for the knockdown of β-catenin. Anti-tubulin antibody was used as a control. (**C**) RT-PCR analysis of CRP mRNA expression following shRNA transfection to HepG2 cells. After 72 h, cells were incubated with or without TNF-α for 6 h. PCR bands were scanned with a densitometer and the density of the CRP band was normalized with that of GAPDH band and is shown graphically. Relative density was presented in comparison to that of vector-transfected cells without TNF-α treatment. Results are the average of three independent experiments.

The NF-κB p50 subunit interacts with β-catenin on the CRP promoter

There is some evidence that the p65 subunit of NF-κB interacts physically with β-catenin (23,24). We tested whether the p50 subunit interacts with β-catenin in the course of TNF-α-induced CRP transcription. We asked if TNF-α promoted the interaction between β-catenin and p50 *in vivo* by performing co-immunoprecipitation assays with FLAG-tagged p50-transfected 293T cells. Even though there is some basal protein–protein interaction between p50 and β-catenin, we found that one hour of TNF-α treatment greatly increased the amount of β-catenin which was co-immunoprecipitated by anti-FLAG antibody (Figure 3A). Since we had shown that TNF-α activates CRP expression by NF-κB p50 on the CRP promoter, we tested if TNF-α causes recruitment of p50 to the -125 CRP promoter. We performed chromatin immunoprecipitation (ChIP) assays with anti-p50 antibody and primers specific for the -125 CRP promoter. As shown in Figure 3B, binding of p50 to the -125 CRP promoter was induced by TNF-α treatment. These results indicate that the interaction between p50 and β-catenin is critical for TNF-α-induced CRP gene transcription.

TNF-α-induced proximity of long distance fragments to CRP promoter

Chromosome conformation capture (3C) is a newly developed technique that can effectively map the long distance interactions between promoter and regulatory sites (14,15,25,26). Since most activation of gene expression involves changes of chromosome conformation, we used the 3C technique to map TNF-α-induced chromosome interaction sites near the CRP locus. 3C uses sequential nuclear restriction enzyme digestion, ligation and PCR-based quantification of the ligated DNA products to assess the proximity of different regions of a chromosome. Two restriction fragments that are close together in the nucleus display a higher cross-linking frequency and therefore a higher ligation frequency than two fragments that are far apart.

Figure 4A shows the CRP locus and the BglII restriction sites within 50 kb of the locus. BglII was chosen because its sites are evenly spaced along the CRP gene and flanking sequences. The segment between fragments 7 and 8 contains the CRP promoter to which NF-κB p50 binds. To test the effect of TNF-α on the proximity of regulatory elements to the CRP promoter, we used an upstream anchor primer within the promoter fragment in pairwise combinations with primers within the fragments of the CRP gene (Figure 4B). All the PCR reactions were performed in triplicate and averaged. The 3C analysis showed that, in the absence of TNF-α (open bar), the proximity of each fragment (fragments 1–7) to the CRP promoter decreased with distance. However, after TNF-α treatment (solid bars), the proximity of fragment 4 to the promoter increased (Figure 4C). We then used the downstream anchor primer in pairwise

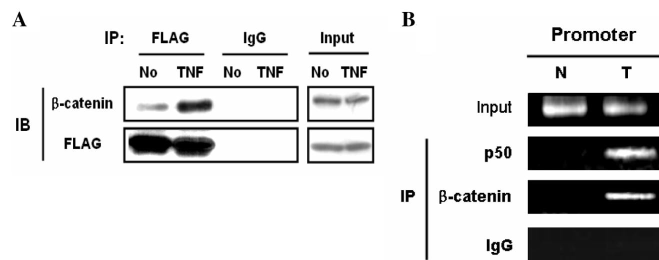

Figure 3. NF-κB p50 and β-catenin interact on the CRP promoter. (**A**) Co-immunoprecipitation assay. Cells of 293T were transfected with a FLAG-tagged p50 expression construct. After 24 h, cells were incubated with or without TNF-α for 1 h. Whole cell extracts were prepared, immunoprecipitated (IP) with anti-FLAG antibody, and the precipitated proteins analyzed by immunoblotting (IB) with anti-β-catenin or anti-FLAG antibody. Input: 8% of whole cell extract. Precipitation with IgG was used as a control. (**B**) Chromatin immunoprecipitation assays with anti-p50 and anti-β-catenin antibodies and the -125 CRP promoter primer. Cells of 293T were treated with TNF-α for 1 h and analyzed the binding of p50 and β-catenin to the CRP promoter.

combination with primers within the BglII fragments and found that TNF-α treatment (solid bar) greatly increased the proximity of fragment 12 (Figure 4D and E). All the 3C data were normalized by the control BAC template and a GAPDH interaction. These results suggest that TNF-α treatment brings fragments 4 and 12 in contact with the CRP promoter.

β-Catenin binds to the downstream region of CRP gene

The induced proximity of chromosomal regions may be caused by interactions between proteins bound to chromosomal sites that are far apart (26). We hypothesized that the 3C interaction observed after TNF-α treatment was due to interaction between p50 bound to the -125 CRP promoter (Figure 3B) and β-catenin bound to the distant regulatory region(s). Since β-catenin itself does not bind to DNA but forms a DNA-bound complex with TCF-4 (16), we searched for putative TCF/LEF-binding sites within fragments 4 and 12. Chromatin immunoprecipitation assays were performed with a probe specific for fragment 4 (Figure 5A). TNF-α treatment did not increase the binding of β-catenin and TCF-4 within fragment 4, which probably reflects the absence of TCF/LEF-binding sites in this fragment. In contrast, six putative TCF/LEF-binding sites were found within fragment 12, and ChIP assays with primers specific for the second putative TCF/LEF-binding sites within fragment 12 showed that the binding of p50, β-catenin and TCF-4 was greatly increased by TNF-α treatment (Figure 5B). This suggests that the -125 CRP promoter bound p50 and the distal regulatory sites (fragments 12) bound β-catenin are in close proximity. We used fragment 13 as a negative control for the ChIP experiments since the 3C experiment showed that its proximity to the promoter did not increase after TNF-α treatment (Figure 5C).

Since the previous ChIP data suggest that the second putative TCF/LEF-binding site might be responsible for the β-catenin binding to fragment 12, we tested whether the site is responsible for the activation of CRP transcription. We designed the -125 CRP luciferase reporters with the putative TCF/LEF-binding sites from fragment 12 located in the downstream of the gene and named them as -125 CRP Luc-2 and -125 CRP Luc-1-2. Figure 6B showed that the luciferase activities of the -125 CRP Luc reporters containing one or two putative TCF/LEF-binding site were increased to 2-fold in comparison to that of -125 CRP Luc reporter having no TCF/LEF-binding site. Protein levels of expressed p50 and β-catenin was not different in these samples as shown by immunoblotting with anti-FLAG, anti-β-catenin antibody (Figure 6B). This result suggests that CRP transcriptional activity is enhanced by the fragment 12 which has putative TCF/LEF-binding sites.

DISCUSSION

We showed here that after TNF-α treatment NF-κB p50 activates CRP expression by associating with β-catenin/TCF bound to a distant downstream region of the CRP gene. The NF-κB p50 subunit lacks a transactivation domain, but we present evidence for a direct interaction between p50 and β-catenin that could provide the p50 with a transactivation domain after TNF-α signaling. We also have evidence that shRNA-mediated suppression of β-catenin significantly reduces TNF-α-induced CRP expression as well as expression of other p50-dependent genes (Choi *et al.*, unpublished data). These results suggest that TNF-α-induced nuclear translocation of β-catenin could serve as a transcriptional co-activator for the p50 subunit.

There is much evidence that PI3-kinase/Akt activation is involved in nuclear translocation of β-catenin following GSK-3β inactivation. It is reported that lipopolysaccharide activates Akt1 in alveolar macrophages resulting in nuclear accumulation and transcriptional activation of β-catenin (27). Just as Wnt-activated β-catenin functions as a transcription co-activator for TCF/LEF proteins, activation of β-catenin by PI3-Kinase/Akt is reported to provide a transcriptional co-activator function for the androgen receptor (28). We also showed here that β-catenin is involved in the activation of CRP gene with NF-κB p50 protein. If nuclear β-catenin plays a role as a transcriptional co-activator for p50, it has to directly or indirectly interact with the p50 subunit. Several reports have suggested a possible relation between β-catenin and NF-κB (23,24,29) but there has been no definitive evidence that TNF-α induced interaction between them. We showed here for the first time that TNF-α significantly increased the extent of interaction between β-catenin and NF-κB p50 subunit. Full understanding of TNF-α-induced CRP transcription may require the identification of the intermediate proteins as well as signal pathways leading to the strong interaction between NF-κB p50 and β-catenin (30).

Transcription is mediated by the binding of specific transcription factors to regulatory sequences. Long-range interactions between promoter and regulatory elements are important for high level expression of target

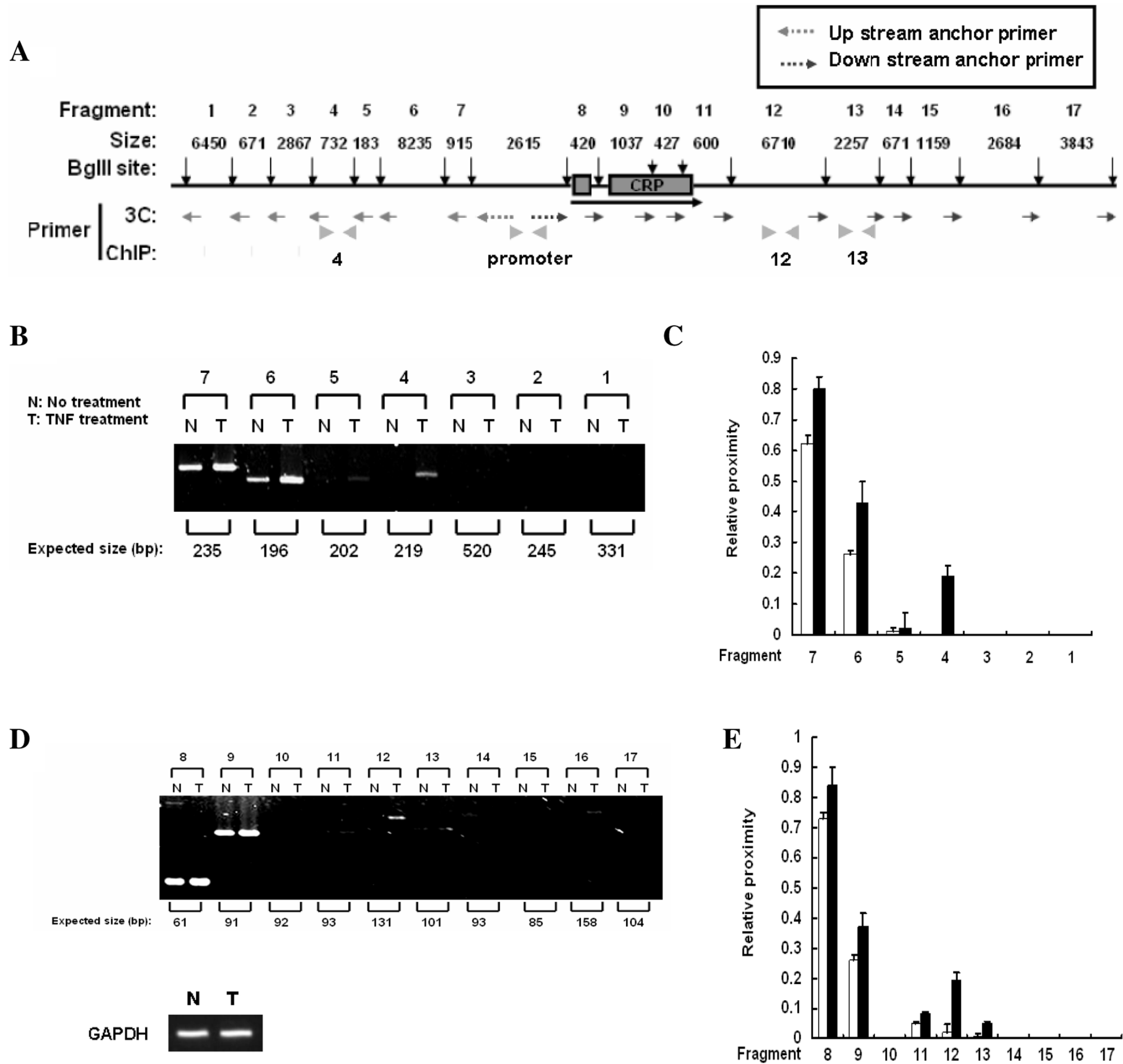

Figure 4. 3C reveals putative chromosome interaction sites near the CRP gene. (**A**) Schematic representation of the CRP locus. CRP locus fragments generated by BglII digestion of genomic DNA and used for 3C analysis are numbered. The fragment containing the CRP promoter is also indicated. Vertical arrows depict the individual BglII sites and the sizes indicate the distances between individual BglII sites. The CRP gene (CRP) is indicated by the boxes (exons) and the line between them (intron), and the orientation of the gene is depicted by the arrows below. The primers used for the 3C and ChIP analysis are also indicated. (**B**) 3C analysis of the upstream regions of the CRP gene. Numbers denote the 3C-analyzed BglII fragments. N; No treated cells,T; TNF-α-treated cells. (**C**) Graphical representation of 3C data for (B). Each signal was normalized to control BAC templates and a GAPDH interaction. (**D**) 3C analysis of the downstream regions of the CRP gene. Numbers denote the 3C-analyzed BglII fragments. N; No treated cells, T; TNF-α-treated cells. (**E**) Graphical representation of 3C data for (D). Each signal was normalized to control BAC templates and a GAPDH interaction. No; Untreated cells (Open box). TNF; TNF-α-treated cells (Solid box).

genes, and may cause looping of chromatin as well as protein–protein interactions (31–33). Since nuclear β-catenin is engaged in TCF/LEF protein complex (34), we screened the genomic sites induced to interact with CRP promoter by 3C technology. We also searched for the TCF/LEF-binding sites and identified a fragment 12 (~3.3 kb downstream from the promoter) as the binding site of β-catenin by ChIP analysis. If nuclear β-catenin binds TCF/LEF which already occupies a specific sequence in fragment 12, it is likely that TNF-induced binding of β-catenin to the CRP promoter is caused by the looping of chromatin between promoter and the TCF/LEF-binding elements in fragment 12.

Our 3C data indicate that the proximity of fragments 4 and 12 to the CRP promoter was increased by TNF-α treatment. However, putative TCF/LEF-binding sites do not exist within fragment 4 and we could not detect any TNF-α-induced binding of β-catenin to fragment 4 by ChIP analysis. This suggests that the interaction between fragment 4 and the CRP promoter may be caused by interaction of p50 with some other protein.

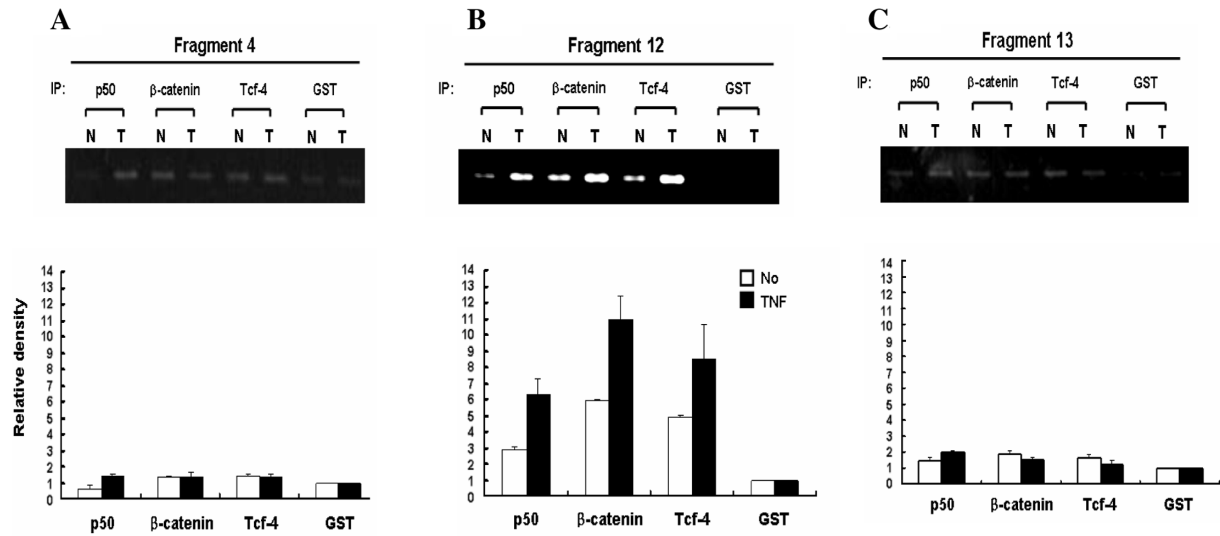

Figure 5. TNF-α-induced β-catenin occupancy on fragment 12. Chromatin immunoprecipitation assay with a fragment 4 primer (**A**) fragment 12 primer (**B**) and fragment 13 primer (**C**). PCR bands were scanned with a densitometer and the densities of p50 and β-catenin-bound bands relative to GST bands are shown graphically. Open box: untreated cells (No). Solid box: TNF-α-treated cells (TNF). Results are the average of three independent experiments.

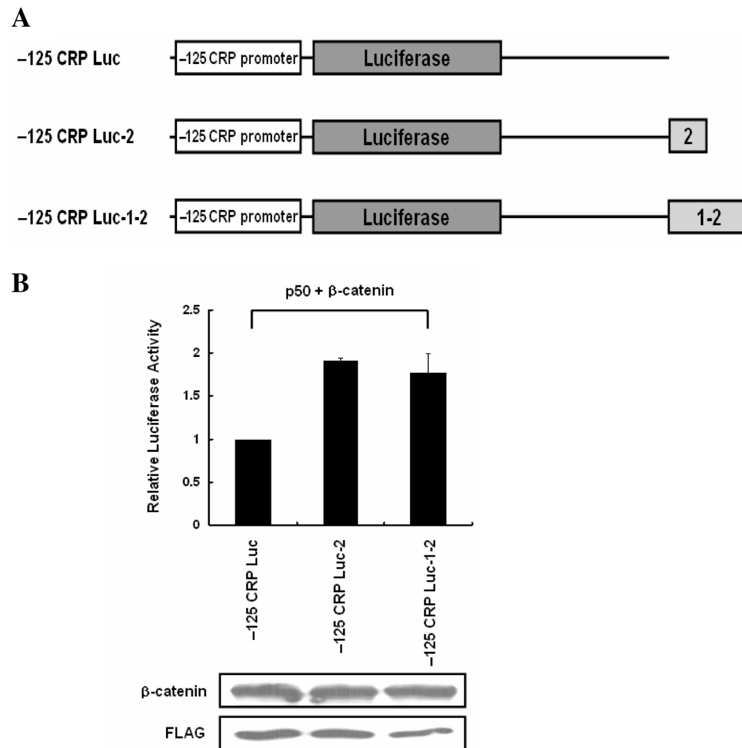

Figure 6. The second putative TCF/LEF-binding site within fragment 12 enhanced the CRP transcription. (**A**) Schematic representation of the constructs with or without putative TCF/LEF-binding sites. -125 CRP Luc (no TCF site); -125 CRP Luc (with second putative TCF/LEF site, shown as 2; -125 CRP Luc (with first and second putative TCF/LEF site, as shown as 1-2). (**B**) Cells were co-transfected with either -125 CRP Luc reporters or NF-κB p50 and stable β-catenin expression vectors. After 24 h, relative luciferase activities were determined. Results are the average of three independent experiments. Western blot analyses for the expression of NF-κB p50 and stable β-catenin were also shown. Expression of p50 and β-catenin was analyzed with anti-FLAG and anti-β-catenin antibody.

Since NF-κB p50 protein may be involved in regulating inflammatory genes such as CRP, we propose here that during TNF-α-induced inflammation, NF-κB p50 induces inflammatory genes by interacting with β-catenin bound to downstream region of the gene. This idea may provide a missing piece of the puzzle posed by the complex cellular NF-κB/β-catenin regulatory network.

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Conflict of interest statement. None declared.

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