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Modulation of the expression of the invasion-suppressor CRMP-1 by cyclooxygenase-2 inhibition via reciprocal regulation of Sp1 and C/EBPα

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Abstract

Collapsin response mediator protein-1 (CRMP-1) controls neural development and axonal growth but also acts as a cancer invasion suppressor. In this study, we investigated the transcriptional regulation of CRMP-1 expression. Using a serial deletion strategy, we identified a basal promoter region between nucleotides -100 and -180 in the 5′ flanking region of *CRMP-1* (nucleotides -1,920 to +50) that contains multiple putative Sp1 and C/EBPα sites. Site-directed mutagenesis and deletion analysis revealed that the two C/EBPα sites, from nucleotides -122 to -133 and from nucleotides -101 to -113, are the most important regulatory elements. Gel-shift and antibody supershift assays showed that Sp1 protein was also present at this C/EBPα site, which overlaps with a Sp1 site. Overexpression of Sp1 decreased *CRMP-1* promoter activity and protein expression, whereas overexpression of C/EBPα produced the opposite effect. Chromatin immunoprecipitation assays confirmed that Sp1 and C/EBPα compete for binding at the overlapping C/EBPα and Sp1 sites and reciprocally regulate *CRMP-1* expression. Overexpression of cyclooxygenase-2 (COX-2) decreased *CRMP-1* mRNA and protein expression. Conversely, the COX-2 inhibitor, celecoxib, induced a dose-dependent increase in *CRMP-1* expression. COX-2 inhibition also decreased Sp1-DNA complex formation and inhibited cell invasion. We conclude that transcription of the invasion suppressor, *CRMP-1*, is reciprocally regulated at the promoter region by C/EBPα and Sp1. COX-2 inhibitors increase CRMP-1 expression by inhibiting Sp1-DNA complex formation and enhancing DNA binding of C/EBPα at the promoter. [Mol Cancer Ther 2008;7(6):1365–75]

Introduction

Metastasis is the major cause of death in cancer patients. It is a complicated process involving cell proliferation, invasion of the basement membrane and stroma, angiogenesis, and distant spread (1, 2) that is controlled by metastasis-enhancing and metastasis-suppressing gene products (3). Some metastasis-suppressing proteins, such as NM23, BRMS1, KiSS-1, and KAI1, have been identified, but their mechanisms of action have not been established (4–7). We have previously identified collapsin response mediator protein-1 (CRMP-1) as a novel invasion suppressor, showing that reduced CRMP-1 expression is associated with early metastasis and poor survival in lung cancer patients (8–10). Deciphering the transcriptional regulation of endogenous CRMP-1 expression will provide insights into the complex mechanisms of cancer metastasis and assist in the design of new strategies for cancer treatment.

CRMP-1 belongs to the CRMP family (CRMP-1 to CRMP-5), which are primarily expressed in the nervous system during embryogenesis (11, 12) and induce neuronal growth cone collapse through the semaphorin/collapsin pathway (13–15). CRMPs are also required for the signaling of the extracellular lysophosphatidic acid–induced growth cone collapse pathway (16). Phosphorylation of CRMP-1 or CRMP-2 by Rho kinase promotes growth cone collapse through microtubule remodeling (16, 17).

Cyclooxygenase-2 (COX-2) is overexpressed in lung cancer cells and is a prognostic marker for lung cancer (18, 19). COX-2 expression can promote lung cancer cell proliferation, invasion, and angiogenesis. COX-2 inhibitors can suppress cancer development by inducing apoptosis or by inhibiting prostaglandin synthesis, cell cycle progression, angiogenesis, or metastasis (20–22). The activation of metastasis suppressor genes may be a new approach for preventing cancer metastasis; however, the association between COX-2 inhibitors and metastasis suppressor genes has not yet been explored.

In this study, we investigated the transcriptional regulation of endogenous CRMP-1 expression and explored the

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Note: C-C. Wu and J-C. Lin contributed equally to this work. T-M. Hong and P-C. Yang codirected the project and contributed equally.

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potential of COX-2 inhibitors to regulate the expression of the invasion suppressor gene, *CRMP-1*. We identified the basal promoter region of *CRMP-1* and found that COX-2 inhibition stimulates *CRMP-1* transcription by modulating the interaction of the transcriptional regulatory elements, Sp1 and C/EBPα, at the promoter region of *CRMP-1*, and thereby suppresses cancer cell invasion in human lung cancer cells.

Materials and Methods

Cell Lines

The CL1-0 human lung adenocarcinoma cell line was established in our laboratory (8, 23). The DNA sequence for silencing *CRMP-1* was subcloned into pSuper/TK-Hyg vector. The stably knockdown of *CRMP-1* in CL1-0 cells was selected by hygromycin (Clontech). The COX-2 inhibitors, NS-398 and celecoxib, were obtained from Sigma and Pfizer, respectively. Small interfering RNA (siRNA) for Sp1 and C/EBPα were purchased from Invitrogen and Dharmacon, respectively.

Reverse Transcription-PCR

RNA was isolated from cells using the RNA-Bee kit (Tel-Test). Total RNA (1 μg) was used for reverse transcription followed by PCR amplification. The PCR primer set used to amplify human *CRMP-1* was sense primer 5′-ATGCCCTGAGCAGACCTGAAGAGC-3′ and antisense primer 5′-AGTAATGGGTGCCATCGGTCCC-CAG-3′. The expression of G_{β}-like was used as an internal control for RNA quantity; the primer sequences were sense primer 5′-GTATGGAACCTGGCTAACTG-3′ and antisense primer 5′-TACTGATAACTTCTTGCTTC-3′ (24). All PCR products were visualized on an ethidium bromide–stained agarose gels.

Western Blot Analysis

Equal amounts (40 μg) of cell lysate were separated by SDS-PAGE (10% gel) and transferred to polyvinylidene membranes (Millipore). The membranes were probed with antibodies directed against Sp1 and CEBP/α (Santa Cruz Biotechnology), CRMP-1 (produced in our laboratory), COX-2 (Cayman Chemical), and β-actin or α-tubulin (Sigma). Antibodies were diluted in PBS (pH 7.0) containing 0.05% (v/v) Tween 20 and 5% (v/v) dried milk. Blots were incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences). Bound antibodies were visualized by enhanced chemiluminescence staining with autoradiographic detection using Kodak X-Omat Blue film (Perkin-Elmer Life Science).

In vitro Cell Invasion Assay

The invasive activity of transfected clones was examined using a membrane invasion culture system (9). The Transwell membrane was fixed with methanol and then stained with a 50 μg/mL solution of propidium iodide (Sigma). The number of cells on each membrane was counted under a microscope at a magnification of ×50 using the Analytical Imaging Station software package (Imaging Research). Each sample was assayed in triplicate.

Cloning the CRMP-1 5′ Flanking Region

The promoter fragment of the *CRMP-1* gene from nucleotides -1,920 to +50 was amplified with the Roche GC-Rich PCR System (Roche Applied Science) using specific BAC clones (Invitrogen) as the templates. Two oligonucleotide primers were used: 5′-GCTTTGTACCGGC-GAAATCT-3′ and 5′-CCGGGAGGGATAGAGACAC-3′. The sense primer contained a *Xho*I site and the antisense primer contained a *Hin*dIII site. The amplified fragment was digested and then inserted into the pGL3-Basic plasmid (Promega).

Construction of the Reporter Plasmids

To generate 5′ serially deleted reporter plasmids, the original reporter plasmid containing the fragment from nucleotides -1,920 to +50 was digested with restriction endonucleases *Xho*I and *Kpn*I. After phenol-chloroform extraction, the linearized plasmid was partially digested starting from nucleotide -1,920 by treating with exonuclease III at 37°C for different times, producing differentially 5′ deleted fragments. After exonuclease III was inactivated at 68°C, the serially deleted plasmids were incubated with mung bean nuclease for 30 min at 37°C. The resulting plasmids were precipitated and then self-ligated using T4 DNA ligase. All procedures were done using the Exo Mung Bean Deletion Kit according to the manufacturer's instructions (Stratagene).

Site-Directed Mutagenesis

Reporter plasmids containing specific mutations in the response element were constructed using mutated primers in a two-step PCR process. The first PCR was done using a sense primer designed to introduce a mutation (Supplementary Table S1[6]; Fig. 2A). This PCR product was then used as the antisense primer for the second PCR; the sequence 5′-CCTGGTTCGAGCCGAGAGG-3′ or 5′-GCTTT-GTACCGGCGAAATCT-3′ was used as the sense primer. The resulting PCR product was ligated into the pGL3-Basic vector. To insert a duplicated novel element into the pCRMP-1(-99/+50) plasmid, double-stranded oligonucleotides containing two copies of the sequence 5′-GGGAG-GAGCTGT-3′ were ligated into the plasmid.

Transient Transfection and Luciferase Activity

Adherent cells (1×10^5) in six-well plates were transiently transfected with plasmids encoding wild-type and 5′ serially deleted luciferase reporter gene constructs using pSV-β-galactosidase (1 μg) dissolved in 10 μL LipofectAMINE (Invitrogen) as the internal control. Cell extracts were harvested after 36 h using 250 μL lysis buffer (Tropix) per well. To determine luciferase and β-galactosidase activities, cell extracts (20 μL each) were assayed separately using the Luciferase Assay Kit and Galacto-Light Plus System (Tropix). Luciferase activity was measured and analyzed using an FB12 luminometer (Zylux).

[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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RNA Interference

Cells were transfected with indicated concentration of specific siRNA oligonucleotides for *Sp1* (Invitrogen) or *C/EBPα* (Dharmacon) using RNAiFect Transfection Reagent (Qiagen). Gene silencing effect was analyzed after 72-h transfection.

Electrophoretic Mobility Shift Assay and Supershift Assay

CL1-0 nuclear extracts were prepared according to the method described previously (25). The nuclear extracts and a ^{32}P-labeled probe were incubated at room temperature, and the reaction was stopped with gel-loading buffer. For supershift assays, anti-Sp1 and anti-Sp3 antibodies (Santa Cruz Biotechnology) were incubated with the nuclear extracts. The three double-stranded oligonucleotides used were probe A [5′-(GGGAGGAGCTGT)$_2$-3′], probe B [5′-(GCCCCCTCCTCCCGCCC)-3′], and probe C (the promoter region from nucleotides -137 to -97).

DNA-Binding Protein Assay

Nuclear extracts (100 μg) for each sample were used after dilution in a total volume of 500 μL DNA-binding protein assay buffer [20 mmol/L HEPES (pH 7.9), 80 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 10% glycerol, and 0.1% Triton X-100] at 30°C for 1 h. The diluted sample was incubated with 50 μL streptavidin paramagnetic beads (Dynal Biotech) at 4°C for 30 min. After the beads were washed three times with DNA-binding protein assay buffer, they were mixed with 20 μL SDS loading buffer and analyzed by immunoblotting.

Chromatin Immunoprecipitation

Chromatin and proteins in CL1-0 cells were cross-linked using 1% formaldehyde, and the reaction was stopped by addition of 0.125 mol/L glycine. The cells were then lysed in 250 μL cell lysis buffer [5 mmol/L HEPES (pH 8.0), 85 mmol/L KCl, 0.5% NP-40, and 1 mmol/L phenylmethylsulfonyl fluoride], and nuclei were collected by centrifugation at 5,000 rpm for 20 min at 4°C. The pelleted nuclei were suspended in 150 μL nuclear lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 1% SDS, 1 mmol/L DTT, and 1 mmol/L proteinase inhibitor], diluted with immunoprecipitation dilution buffer [0.01% SDS, 1% Triton X-100, 1 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.0), 167 mmol/L NaCl, 1 mmol/L DTT, and 1 mmol/L proteinase inhibitor] and sonicated at 12 W for 45 s. The DNA/protein-containing supernatant was incubated with anti-Sp1 and anti-C/EBPα antibodies at 4°C overnight. The immunoprecipitated complexes were collected using protein A-Sepharose beads and then washed sequentially with low-salt buffer [0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% deoxycholic acid], high-salt buffer [0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 300 mmol/L NaCl, and 1% deoxycholic acid], LiCl buffer [300 mmol/L LiCl, 1% Triton X-100, 0.01% SDS, 1 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% deoxycholic acid], and Tris-EDTA. The precipitates were eluted with elution buffer (1% SDS and 100 mmol/L $NaHCO_3$). To reverse the cross-linking, 5 mol/L NaCl and RNase were added, and the samples were incubated overnight at 65°C. The extracted DNA was analyzed by PCR using primers spanning the proximal (nucleotides -180 to -161) or distal (nucleotides +32 to +50) region of human *CRMP-1*. After 45 cycles of amplification, the PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Results

Mapping the Endogenous Response Element in the CRMP-1 Promoter

To investigate the transcriptional regulation of the human *CRMP-1* gene, genomic DNA fragments containing the human *CRMP-1* promoter region from nucleotides -1,920 to +50 were cloned into the pGL3-Basic vector. A series of nine deletion mutants was generated to identify the relevant region of the *CRMP-1* promoter (Fig. 1A). The reporter plasmid pCRMP-1(-99/+50) containing the smallest promoter fragment displayed ~7-fold greater transcriptional activity than did the pGL3-Basic plasmid. The transcriptional activity of the reporter plasmid containing nucleotides -1,920/+50 to -180/+50 fragment was substantially greater: 35- to 60-fold that of the control plasmid. Intriguingly, deletion of the promoter region from nucleotides -180 to -100 produced a dramatic, ~7-fold reduction in transcriptional activity relative to the full-length, nucleotides -1,920 to +50 fragment, indicating that this region contained a response element that was critical for the regulation of *CRMP-1* expression. Based on computer predictions,[7] this region contains three Sp1 sites and two inverted, putative C/EBPα-binding sites (Fig. 1B; ref. 26).

Identification of C*is*-Acting Sp1 and C/EBPα Regulatory Elements in the CRMP-1 Promoter by Site-Directed Mutagenesis

To evaluate the functional importance of these elements, we constructed a series of plasmids containing site-directed mutations in Sp1- and C/EBPα-binding sites and evaluated their transcriptional activities in luciferase assays (Fig. 2A). Mutation of the Sp1-binding site and second C/EBPα-binding site (pCRMP-1 M9 and M10, respectively) in nucleotides -180 to +50 fragment had no effect on human *CRMP-1* transcriptional activity. However, when nucleotides -126 and -127 in the C/EBPα-binding site were mutated from AG to GA (pCRMP-1 M2), the promoter activity was decreased substantially (~60%) compared with that of the pCRMP-1 M1 plasmid. Introduction of this mutation at the corresponding site in nucleotides -1,920 to +50 fragment (pCRMP-1 M11) resulted in a similar decrease in activity.

To determine the length of this response element, we then mutated various sites adjacent to nucleotides -126 and -127 as illustrated in Fig. 2A. Luciferase activity decreased 25% to 50% in the M3, M4, and M5 plasmids containing

[7] http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl

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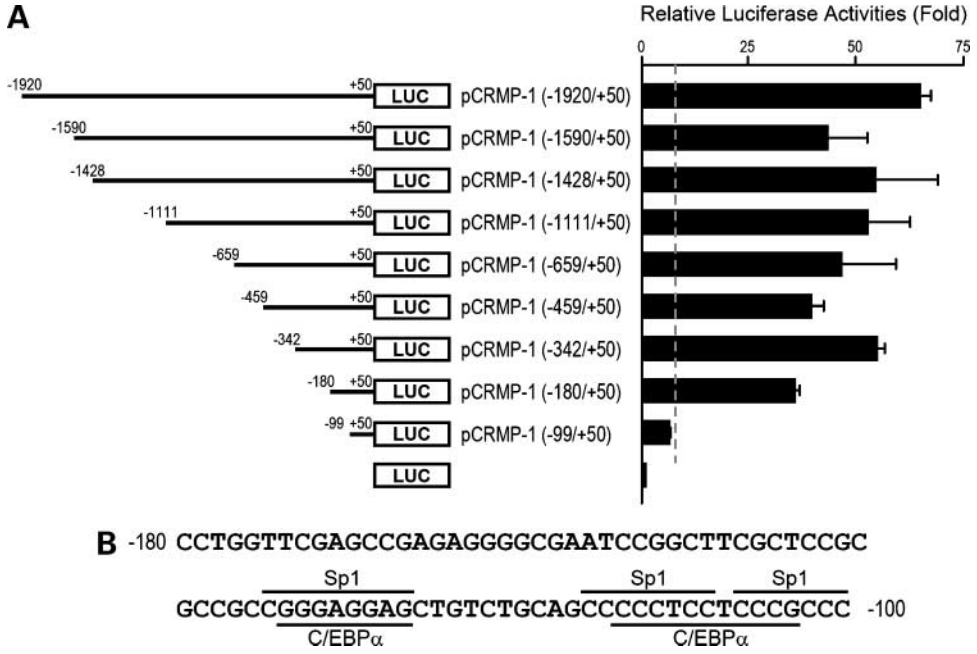

Figure 1. Mapping the response element in the *CRMP-1* promoter. **A,** human *CRMP-1* promoter-luciferase reporter constructs were transfected into CL1-0 cells. The luciferase activity of each construct was then compared with the mean value for the empty pGL3-Basic vector to calculate the fold induction of each construct. **B,** nucleotide sequence of the 5′ flanking region of the human *CRMP-1* gene between nucleotides -99 and -180. There are two putative C/EBPα-binding sites and three Sp1-binding sites as indicated. Mean ± SD.

mutations at nucleotides -131/-130, -122/-121, and -133/-132, respectively, but more distal mutations (M6 and M7 plasmids) had no effect. When the region from nucleotides -180 to -117 was deleted from pCRMP-1(-180/+50), luciferase activity decreased by ~60% relative to the wild-type pCRMP-1 plasmid (Fig. 2B). Similar results were also noted for pCRMP-1(-180/+50) plasmids containing mutations to different nucleotides at -126 and -127. Collectively, our results suggest that the region between nucleotides -122 and -133 (GGGAGGAGCTGT) is crucial for the proximal promoter activity of human *CRMP-1*. Importantly, the element containing the C/EBPα-binding site (from -126 to -133) is located in this region as indicated in Fig. 1B.

When pCRMP-1(-117/+50) was mutated at either one or both of the putative Sp1 sites that overlap the C/EBPα-binding site, there was a modest additional decrease in luciferase activity (~25%): from ~0.4-fold that of nucleotides -180/+50 fragment containing nucleotides -126 to -133 binding site to 0.25-fold. This indicates that the region from nucleotides -100 to -117 makes a relatively minor contribution to overall activity compared with the region from nucleotides -126 to -133 (Fig. 2B). The results obtained with pCRMP-1(-180/+50) plasmids containing only mutations in nucleotides -100 to -117 site (M9 and M10) were also consistent with this interpretation (Fig. 2A). We conclude from these results that the novel binding site at nucleotides -126 to -133 is a significant regulator of human *CRMP-1* promoter activity.

Interestingly, the luciferase activity of the pCRMP-1(-99/+50) plasmid containing two extra GGGAGGAGCTGT repeat sequences was very similar to that of the pCRMP-1(-180/+50) plasmid (Fig. 2C). The duplicate elements within the promoter increased the transcriptional activity ~5-fold compared with that of pCRMP-1(-99/+50). Thus, the C/EBPα-binding sites contribute most of the promoter activity of *CRMP-1*. These results further confirm that the C/EBPα-binding site is a basal element in *CRMP-1* transcriptional regulation.

C/EBPα and Sp1 Are Involved in the Transcriptional Regulation of CRMP-1 Expression

To determine whether Sp1 and C/EBPα proteins bound to these elements, we first prepared three ^{32}P-labeled, double-stranded oligonucleotide probes and used electrophoretic mobility shift assay to analyze binding of nuclear proteins. Probe A contained a dual GGGAGGAGCTGT sequence (-122 to -133); probe B corresponded to nucleotides -100 to -117, and probe C corresponded to nucleotides -97 to -137 (Fig. 3A). Nuclear extracts were incubated with a 100-fold excess of unlabeled probe. With all probes, three major specific protein-DNA complexes were detected (Fig. 3B). Supershift analyses using antibodies directed against Sp1 and Sp3 identified the upper band in each case as a Sp1-DNA complex (Fig. 3B). To determine whether C/EBPα protein also bound to these elements, the three probes were analyzed using a DNA affinity precipitation assay (Fig. 3C). The C/EBPα protein also bound to all three probes.

Negative Regulation by Sp1 and Positive Regulation by C/EBPα at the CRMP-1 Promoter

To confirm the involvement of Sp1 and C/EBPα in regulating the *CRMP-1* promoter, we coexpressed Sp1 or C/EBPα proteins with luciferase reporter constructs in the CL1-0 cell line. Overexpression of Sp1 decreased the luciferase activity of almost all plasmid constructs by ~50% (Fig. 4A). The exception was the pCRMP-1(-99/+50) plasmid, which exhibited similar levels of luciferase activity in the presence and absence of overexpressed Sp1. Conversely, overexpression of C/EBPα increased the

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luciferase activity of the pCRMP-1 plasmids, although this increase was attenuated in the case of the pCRMP-1(-99/+50) plasmid (Fig. 4A). These results suggest that the critical region of the *CRMP-1* promoter for Sp1 and C/EBPα binding is located between nucleotides -180 and -100.

To further confirm the roles of Sp1 and C/EBPα in regulating *CRMP-1* expression, we exogenously expressed Sp1 and C/EBPα transiently in CL1-0 cells and examined levels of endogenous CRMP-1 protein. The level of CRMP-1 protein decreased on overexpression of Sp1 and increased with C/EBPα overexpression (Fig. 4B). Sp1-induced decreases and C/EBPα-induced increases in CRMP-1 were largely reversed when Sp1 or C/EBPα expression was silenced by specific siRNA (Fig. 4C). These findings indicate that Sp1 protein acts as a negative regulator and C/EBPα protein as a positive regulator at the *CRMP-1* promoter, suppressing and enhancing *CRMP-1* transcription, respectively.

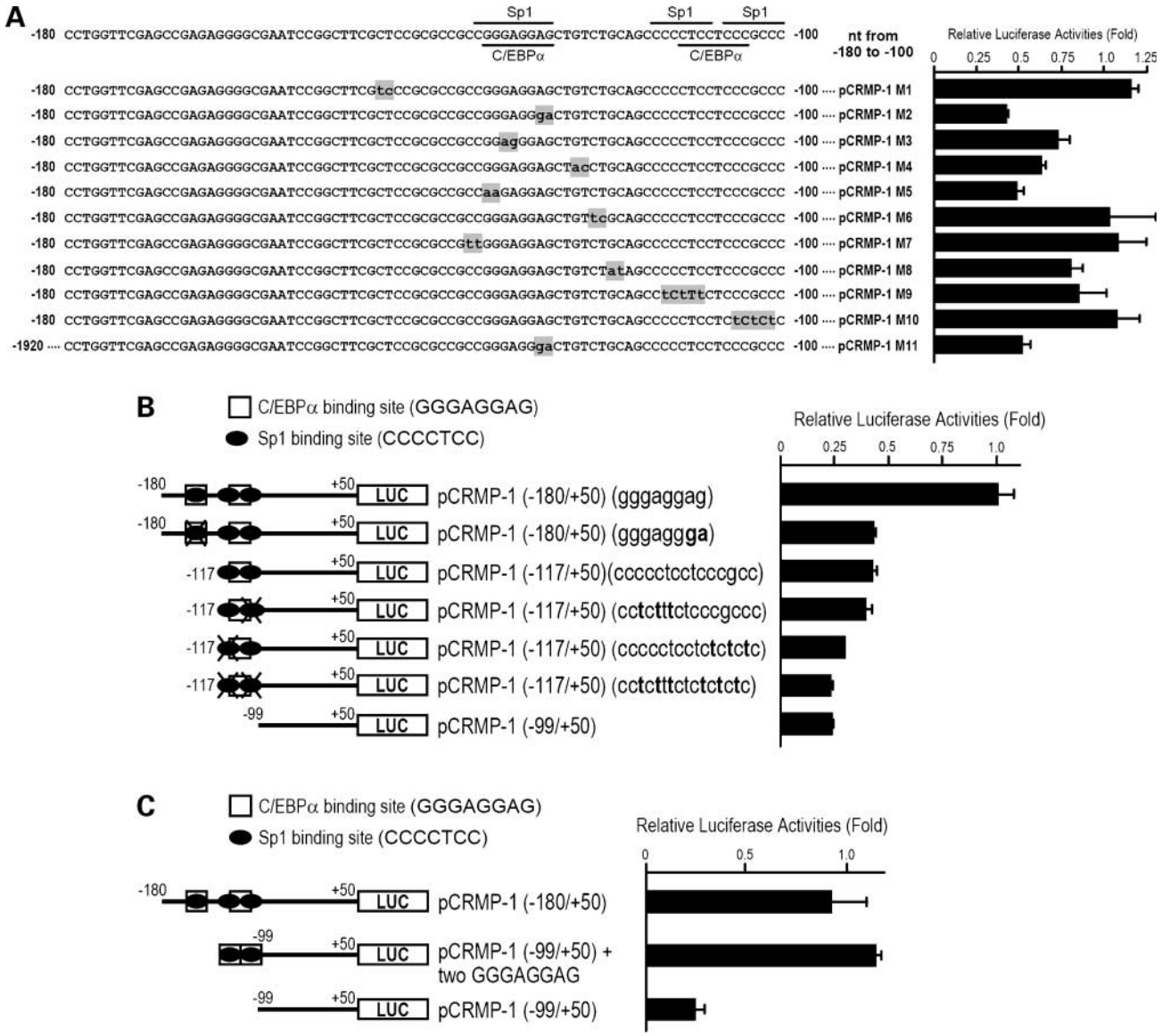

Figure 2. Mutational analysis of *CRMP-1* promoter-luciferase constructs. **A,** the reporter construct, pCRMP-1(-180/+50), was individually mutated at different sites. *Gray boxes,* mutation sites in pCRMP-1 mutated plasmids (M1-M10). Luciferase activity for each mutant plasmid was expressed relative to the mean value for pCRMP-1(-180/+50) to calculate fold activity. The reporter construct pCRMP-1(-1920/+50) was mutated to produce pCRMP-1 M11. The luciferase activity of pCRMP-1 M11 was expressed relative to the mean value for pCRMP-1(1920/+50). **B,** deletions were made in the reporter construct pCRMP-1(-180/+50) to create the reporter construct, pCRMP-1(-117/+50), which was subsequently mutated at different individual sites. The luciferase activity of each construct was expressed relative to the mean value for pCRMP-1(-180/+50) to calculate fold activity. The mutated sites are represented in bold. **C,** luciferase activity of the reporter construct containing two GGGAGGAGCTGT sequences was expressed relative to the mean value for pCRMP-1(-180/+50) to obtain the fold activity. Mean ± SD.

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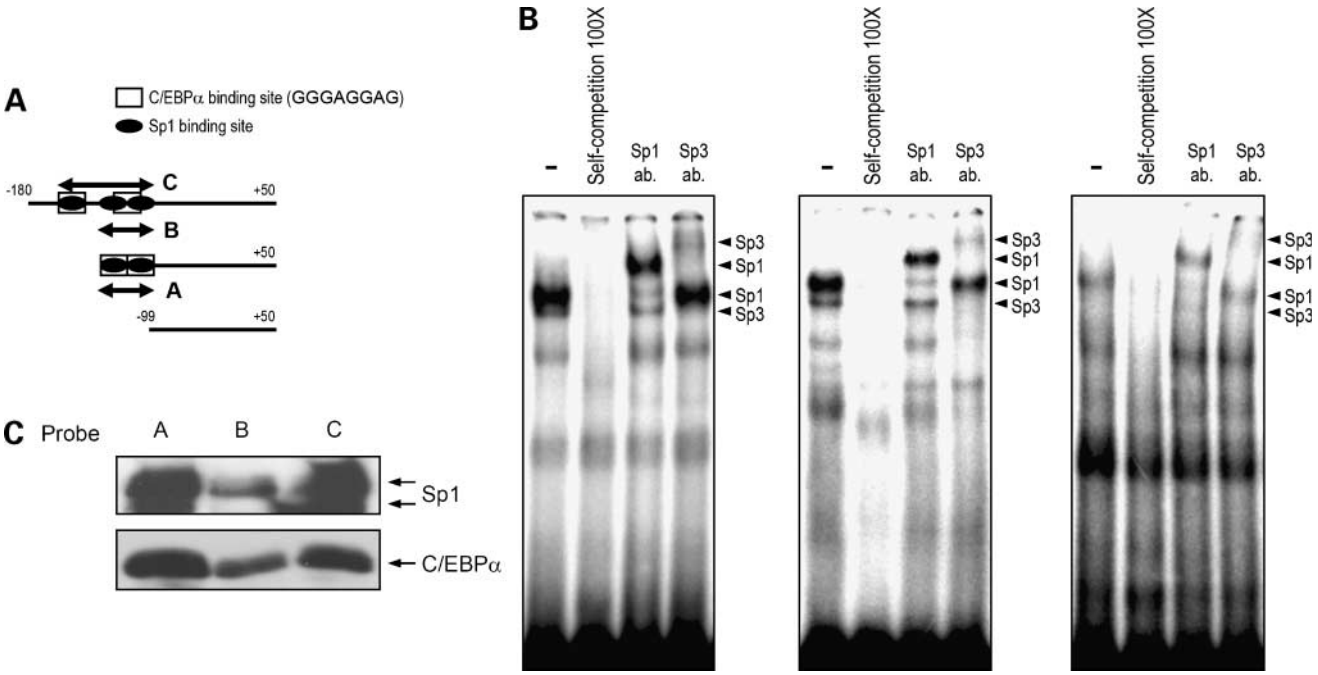

Figure 3. Identification of specific protein complex binding at the *CRMP-1* promoter region. **A,** DNA-binding activity in CL1-0 cell nuclear extracts (2.5 μg) was determined in gel-shift assays using three sequence fragments as probes. Probe A contains two repeats of the GGGAGGAGCTGT sequence; probe B corresponds to nucleotides -117 to -100 sequence; and probe C corresponds to nucleotides -137 to -97 sequence. **B,** electrophoretic mobility shift assays using probes A, B, and C as templates: *lane 1,* positive control (no competing unlabeled probe); *lane 2,* competition with 100-fold excess unlabeled probe; *lanes 3* and *4,* preincubation with Sp1 or Sp3 antibody, respectively. **C,** DNA-binding protein assay of probes A, B, and C using 100 mg CL1-0 nuclear extract. CL1-0 cells were transfected with pSG5-Sp1 or pCMV-C/EBPα plasmids to overexpress Sp1 or C/EBPα proteins, respectively, in the CL1-0 nucleus. The three biotin-labeled probes were incubated with the nuclear extracts and then precipitated with streptavidin paramagnetic beads. The samples were analyzed by Western blotting using anti-Sp1 and anti-C/EBPα antibodies.

COX-2 Inhibition Up-regulates CRMP-1 Expression and Suppresses Cancer Cell Invasion

COX-2 is overexpressed in lung cancer cells and may promote tumor proliferation, invasion, and angiogenesis (24, 27, 28). To assess the relationship between COX-2 and CRMP-1 in cancer metastasis, we exogenously expressed COX-2 transiently in CL1-0 cells. The results indicate that *CRMP-1* mRNA and CRMP-1 protein expression was suppressed by COX-2 in a dose-dependent manner. The results from luciferase reporter assays localized the major COX-2-responsive element to nucleotides -180 to -100 promoter region (Fig. 5A). To determine the effect of COX-2 inhibition on endogenous *CRMP-1* expression, we treated CL1-0 cells with the COX-2 inhibitors, NS-398 and celecoxib (0-50 μmol/L), and assayed the levels of *CRMP-1* mRNA and CRMP-1 protein by reverse transcription-PCR and Western blotting, respectively. Both *CRMP-1* mRNA and CRMP-1 protein levels increased in a dose-dependent manner with increasing concentrations of COX-2 inhibitors. The level of C/EBPα also increased in the presence of COX-inhibitors, but Sp1 levels decreased (Fig. 5B and C). To show a link among COX-2 activity, CRMP-1 modulation, and altered invasivity, we analyzed the invasive ability of COX-2 inhibitor-treated cells after knocking down CRMP-1 expression using siRNA gene silencing. In the absence of siRNA, celecoxib suppressed cancer cell invasion in a dose-dependent manner. Silencing of *CRMP-1* enhanced the invasivity of untreated cancer cells, as expected based on our previous demonstration of the role of CRMP-1 as an invasion suppressor, but treatment with celecoxib did not completely suppress invasion to basal levels (Fig. 5D). These results suggest that up-regulation of CRMP-1 expression may at least partially explain the suppression of cancer cell invasivity by COX-2 inhibitors.

COX-2 Inhibition Suppresses Sp1-DNA Complex Formation and Enhances C/EBPα-DNA Binding at the CRMP-1 Promoter

To examine the effect of the COX-2 inhibitors on *CRMP-1* expression, we measured the activity of reporters containing the *CRMP-1* promoter region by luciferase assay after celecoxib treatment. Luciferase activity was enhanced 1.8-fold in constructs containing promoter sequences from nucleotides -180 to +50 [pCRMP-1(-180/+50); Fig. 6A], indicating that the COX-2 inhibitor modulates *CRMP-1* expression at the transcriptional level. To determine whether the COX-2 inhibitor enhances CRMP-1 expression by regulating the binding activities of Sp1 and C/EBPα at the *CRMP-1* promoter, we monitored changes in the DNA-binding ability of Sp1 and C/EBPα at the critical nucleotides -180 to -100 element of the *CRMP-1* promoter

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in cells treated with NS-398. Electrophoretic mobility shift assay showed a gradual decrease in the Sp1-DNA complex band after cells were treated with NS-398 at concentrations of 25 and 50 μmol/L (Fig. 6B). Chromatin immunoprecipitation assays also confirmed that NS-398 at the same concentrations reduced the binding affinity of Sp1 for DNA at this critical element of the *CRMP-1* promoter while enhancing that of C/EBPα. Thus, COX-2 inhibition produced opposite effects on Sp1 and C/EBPα binding to the *CRMP-1* promoter (Fig. 6C).

Overall, C/EBPα binding to its regulatory element in the *CRMP-1* promoter increased *CRMP-1* expression (Fig. 6D.1). In contrast, when Sp1 bound to its binding site, *CRMP-1* expression was suppressed (Fig. 6D.2). After treatment with the COX-2 inhibitor, Sp1 binding to DNA was inhibited, allowing C/EBPα to bind more readily to the *CRMP-1* promoter and stimulate CRMP-1 expression (Fig. 6D.3).

Discussion

We identified previously that CRMP-1 is an invasion and metastasis suppressor in lung cancer cells (9). CRMP-1 expression is negatively associated with poor overall survival and early postoperative relapse in lung cancer patients (9). The connective tissue growth factor can inhibit

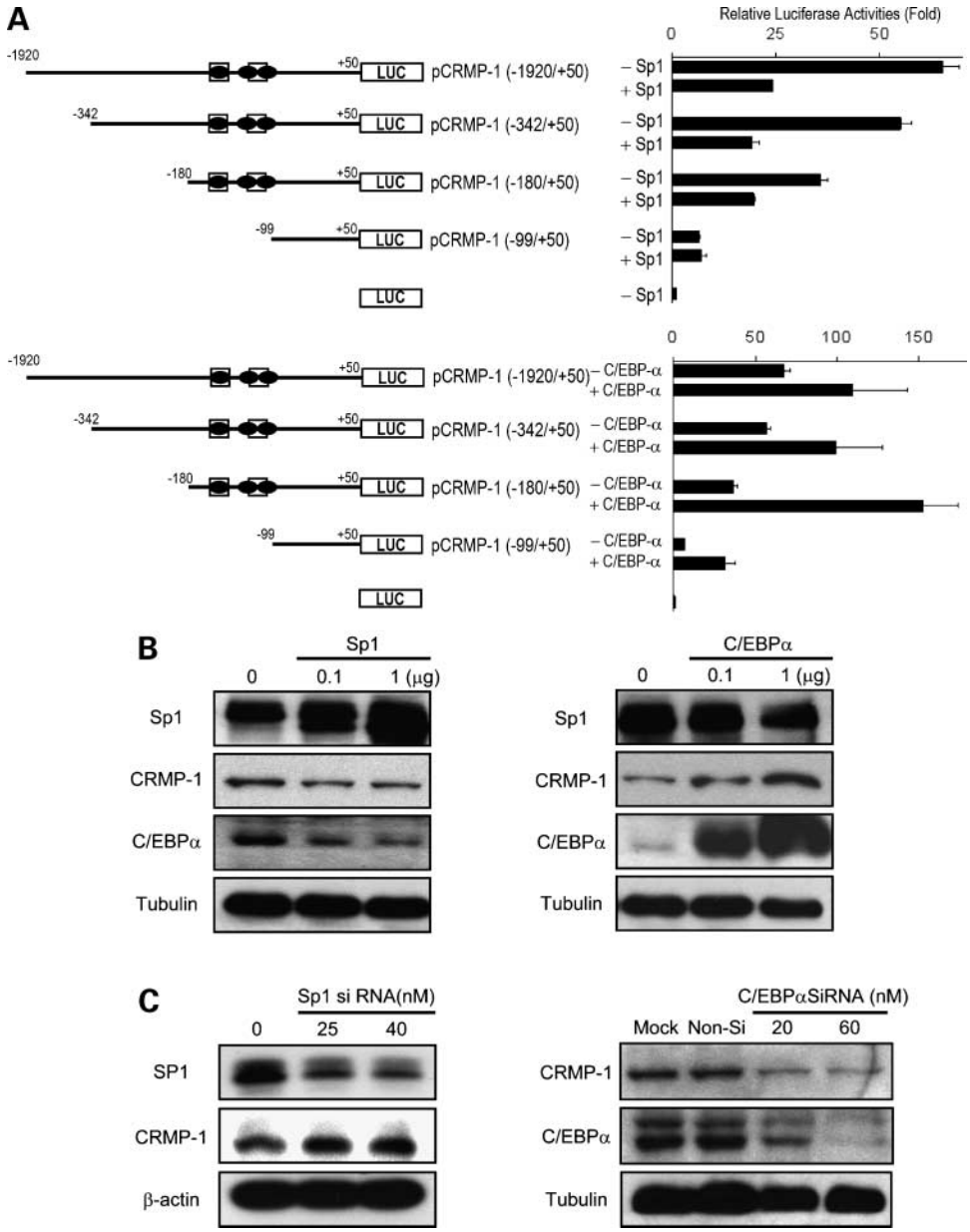

Figure 4. Suppressing and enhancing effects of Sp1 and C/EBPα on the *CRMP-1* promoter in lung cancer cells. **A,** CL1-0 cells, transfected with 1 μg pSG5-Sp1 or pCMV-C/EBPα,were cotransfected with the various constructs as indicated. Cells transfected with pSG5 and pCMV vectors were included as controls. Luciferase activity was measured 36 h after transfection. Mean ± SD. **B,** effect of overexpressing Sp1 and C/EBPα protein on CRMP-1 expression. CL1-0 cells were transfected with 0.1 and 1 μg pSG5-Sp1 or pCMV-C/EBPα or vector controls. CRMP-1, Sp1, C/EBPα, and tubulin (internal control) protein levels were determined by Western blotting. **C,** effect of siRNA-mediated silencing of Sp1 or C/EBPα on CRMP-1 expression. CL1-0 cells were treated with the indicated amounts of siRNA for Sp1 or C/EBPα. CRMP-1, Sp1, C/EBPα, and tubulin (internal control) protein levels were determined by Western blotting.

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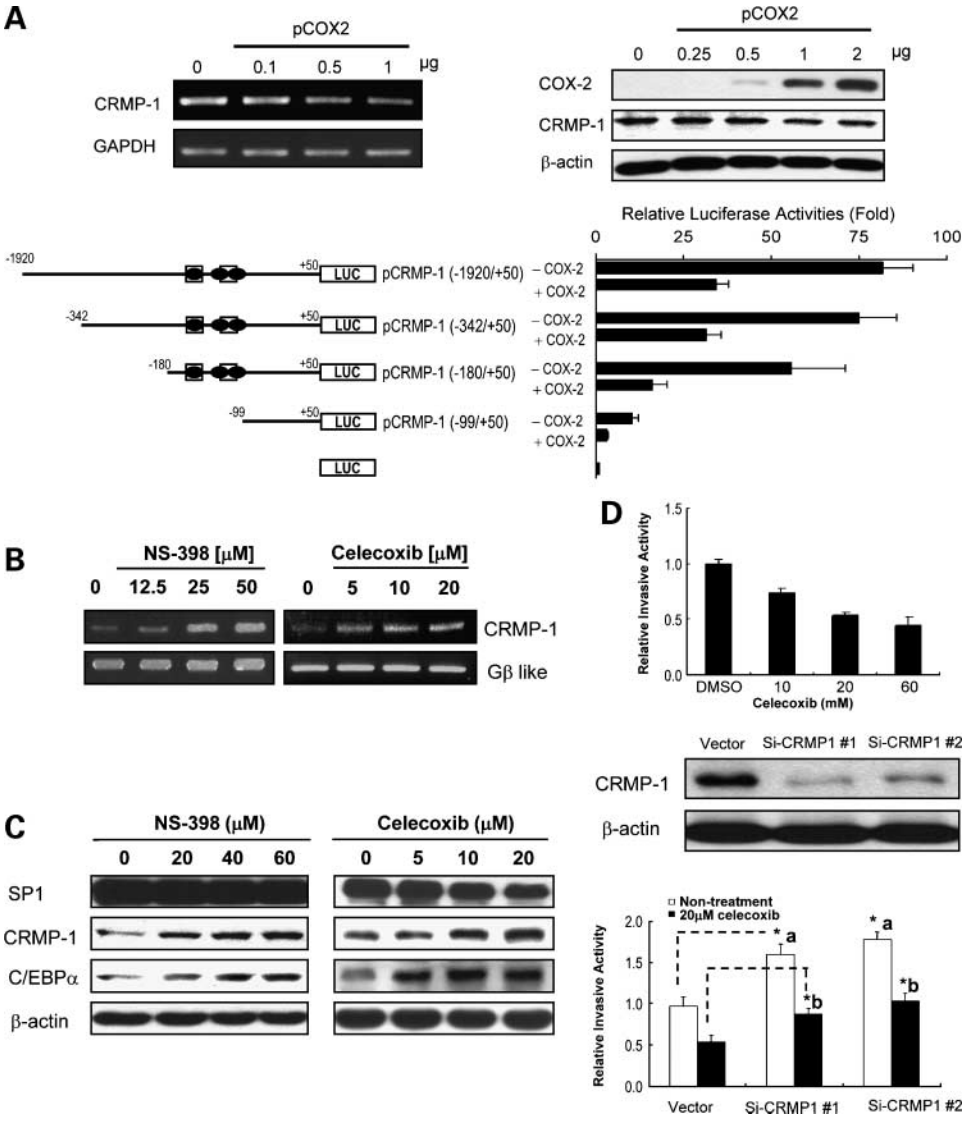

Figure 5. Up-regulation of *CRMP-1* expression in lung cancer cells by COX-2 inhibitors. **A,** CL1-0 cells were transfected with increasing amounts of pSG5-COX-2 plasmids as indicated. After 24 h, the levels of *CRMP-1* mRNA and protein were analyzed by reverse transcription-PCR and Western blotting, respectively; glyceraldehyde-3-phosphate dehydrogenase and β-actin were used as internal controls. Luciferase activity was measured after 36 h and expressed relative to β-galactosidase activity. Mean ± SD. **B,** CL1-0 cells were treated with increasing concentrations of the COX-2 inhibitors, NS-398 and celecoxib. *CRMP-1* mRNA (**C**) and CRMP-1 protein (**D**) were analyzed by reverse transcription-PCR and Western blotting, respectively. The level of Sp1 protein decreased slightly, but CRMP-1 and C/EBPα protein levels increased in dose-dependent manner. **D,** CL1-0 cells were treated with the indicated concentrations of celecoxib. Cell invasion was measured after 24 h using a Matrigel invasion culture system (*top*). The invasion activities of cells stably expressing CRMP-1 siRNA or vector control, as indicated, were assayed after 20 μmol/L celecoxib treatment (*bottom*). **a* and **b, P* < 0.05, statistically significance differences between vector control and CRMP-1-silenced groups treated without and with 20 μmol/L celecoxib, respectively.

lung adenocarcinoma invasion and metastasis by CRMP-1-dependent pathway and mediated through an integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ regulated signaling (29). However, the detail mechanism of invasion suppressor function of CRMP-1 and its regulation were still not clear.

In this study, we identified a region between nucleotides -100 and -180 containing putative Sp1 and C/EBPα transcriptional regulatory elements as the basal promoter region of the novel invasion suppressor gene, *CRMP-1*. Overexpression of Sp1 suppressed *CRMP-1* promoter activity and CRMP-1 protein expression, whereas overexpression of C/EBPα enhanced expression from the *CRMP-1* promoter. Our data also showed that COX-2 overexpression decreased *CRMP-1* mRNA and protein expression in human lung cancer cells. Conversely, COX-2 inhibitors induced a dose-dependent increase in *CRMP-1* expression, with a concomitant inhibition of cell invasion. We showed that COX-2 inhibitors up-regulated endogenous *CRMP-1* transcription by suppressing the formation of Sp1-DNA complexes and enhancing the binding of C/EBPα to the *CRMP-1* promoter. These findings may increase our understanding of the mechanism of metastasis suppressor gene regulation and contribute to the development of new therapies targeting metastasis in cancer patients.

The CRMP-1 promoter lacks a TATA box and contains a CpG island, which has several Sp1-binding sites. Our data showed that the key basal regulatory element was located at nucleotides -100 to -180, a region that contains putative Sp1 and C/EBPα transcriptional regulatory elements. Site-directed mutagenesis data (e.g., plasmids M2-M5) indicated that the region between nucleotides -122 and -133 (GGGAGGAGCTGT) was crucial for the proximal promoter activity of human *CRMP-1*. Another important response element was located at nucleotides -100 to -117, which contains a sequence (CCCTCCTC) that is the

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complement of GGGAGGAG, suggesting that this sequence may be an important response element for transcription factors. In addition, we propose that the dimeric form of C/EBPα could bind the two inverted DNA sequences ''GGGAGGAG'' and ''CTCCTCCC'' in nucleotides -133 to -101 region. This interpretation is consistent with the ability of the M4 mutation, which is located outside of the defined C/EBPα-binding sites, to diminish reporter activity. Accordingly, because it lies between the two C/EBPα-binding sites, this mutation may cause structural or conformational changes that interfere with C/EBPα binding but not Sp1 binding. Although the -99/+50 region may contain an additional C/EBPα response element (Fig. 4A), the critical response element for the expression of CRMP-1 corresponds to the -180/-100 region.

In general, Sp1 binds to promoter Sp1 consensus sequences to activate gene expression. However, we found that Sp1 bound to a *cis*-acting regulatory element on the promoter of the metastasis suppressor gene, *CRMP-1*, to down-regulate gene expression. Previous studies have also suggested such a transcriptional repressor role for Sp1 binding in various cells. For example, increasing Sp1 protein and Sp1-binding capacity by stimulation with fibroblast growth factor-2 has been shown to decrease platelet-derived growth factor receptor-α transcription in rat aortic smooth muscle cells (29). Sp1 also inhibits the proliferation of vascular smooth muscle cells by repressing *CDKN1A* (*WAF1/CIP1*) transcription via a *cis*-acting regulatory element in the promoter of the gene (30, 31).

C/EBPα is a leucine-zipper transcription factor. It can occupy the activation response element on the COX-2 promoter and thereby abolish transcriptional activity and suppress COX-2 expression (32). The expression of C/EBPαis reduced in lung adenocarcinomas and at more

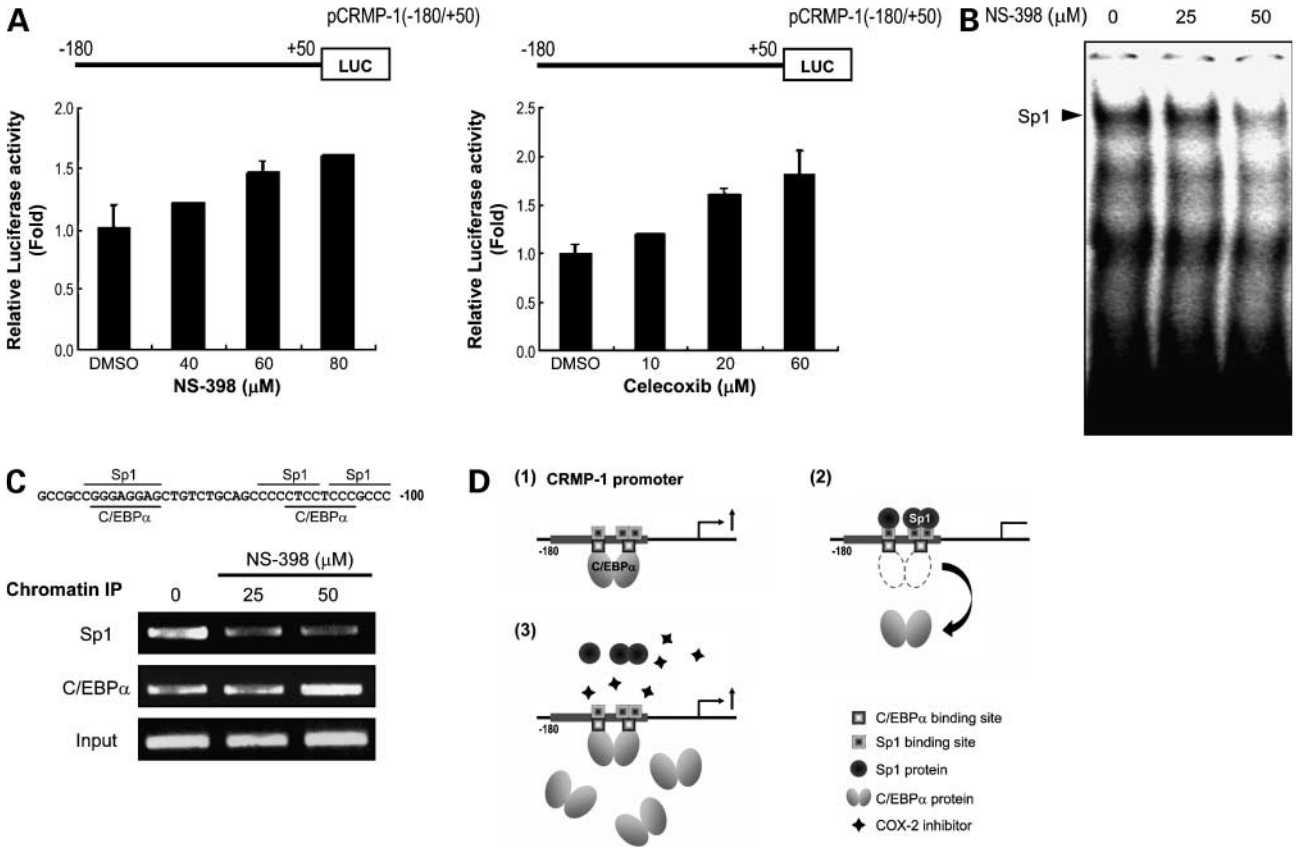

Figure 6. Modulation of CRMP-1 gene expression by COX-2 inhibitors through reciprocal regulation of Sp1 and C/EBPα. **A,** CL1-0 cells transfected with the human *CRMP-1* promoter-luciferase reporter construct (nucleotides -180 to +50) were treated with the indicated concentrations of NS-398 and celecoxib for 36 h. Luciferase activity of treated cells was expressed relative to untreated cells. Mean ± SD. **B,** DNA binding by nuclear proteins extracted from untreated control CL1-0 cells (*lane 1*) or cells treated with 25 μmol/L (*lane 2*) or 50 μmol/L (*lane 3*) NS-398 was analyzed by electrophoretic mobility shift assay. A nucleotide fragment of the CRMP-1 promoter region corresponding to nucleotides -180 to -100 was used as the probe. **C,** chromatin immunoprecipitation assays were done on untreated control CL1-0 cells (*lane 1*) and cells treated with 25 or 50 μmol/L NS-398. The extracted chromatin was immunoprecipitated with anti-Sp1 and anti-C/EBPα antibodies as indicated. Recovered DNA was amplified by PCR using primers covering the region of the *CRMP-1* promoter from nucleotides -180 to +50. Control amplifications were done on preimmunoprecipitated (''input'') chromatin. **D,** model summarizing COX-2 inhibitor affects on CRMP-1 expression through Sp1 and C/EBPα. (*1*) C/EBPα binding to the *CRMP-1* promoter at the C/EBPα-binding site stimulates *CRMP-1* gene expression. (*2*) Sp1 competition with C/EBPα at overlapping C/EBPα-Sp1 binding sites down-regulates *CRMP-1* expression. (*3*) After treatment with a COX-2 inhibitor, Sp1 cannot bind to the *CRMP-1* promoter, allowing C/EBPα to bind more readily to the *CRMP-1* promoter. Thus, *CRMP-1* expression is up-regulated.

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advanced stages in other cancers. Same expression pattern was also observed in CRMP-1 in lung cancer tissue. The ectopic expression of C/EBPα in non-small cell lung cancer cell lines leads to reduced growth, increased apoptosis, and cell differentiation (33). C/EBPα up-regulates the tumor suppressor protein, hepatocyte nuclear factor-3β, which plays an important role in airway epithelial differentiation (34). Thus, C/EBPα is a novel tumor suppressor protein that can trigger tumor suppressor-related gene expression in lung cancer. Our observation that COX-2 inhibition can increase C/EBPα protein expression and up-regulate CRMP-1 expression suggests that C/EBPα may play a role in countering cancer cell invasivity through CRMP-1 pathway.

It is often the case that the successful transactivation of a specific gene crucially depends on the precise spacing of two (or more) transcription factor binding sites (35). The lactoferrin gene promoter contains a C/EBPα site flanked by two Sp1 sites, and the interaction of these two transcription factors is required to activate gene expression (36). This DNA sequence motif is similar to that of the *CRMP-1* gene promoter (Fig. 1), which is capable of binding these two transcription factors at its *cis*-acting response element (Fig. 3C). Forced expression of Sp1 or C/EBPα suppressed or induced *CRMP-1* promoter activity, respectively. Sp1 and C/EBPα competed for binding to the overlapping C/EBPα and Sp1 sites and reciprocally regulated *CRMP-1* expression. Tang et al. have shown that the promoter of the mouse C/EBPα gene, *cebpa*, contains a Sp1 consensus sequence, which overlaps with a consensus C/EBP recognition sequence. Further, they showed that Sp1 competes with C/EBP for binding to the coregulatory element and represses *cebpa* promoter activity. Decreasing the level of Sp1 facilitated the access of other C/EBP to the binding site and reactivated *cebpa* gene expression (37).

COX-2 is overexpressed in colon, lung, breast, prostate, esophageal, and pancreatic cancers (2, 18, 37–42) and has been implicated in regulating the malignant phenotype in lung cancer. However, the mechanism by which COX-2 acts to promote cancer invasion is still unclear. Recent reports have shown that prostaglandin E_2, a COX-2 product, induces vascular endothelial growth factor via activation of Sp1 (43). Accumulating evidence suggests that activation of the Sp1 transcription factor—by enhancing the expression of metastasis promoter genes, such as *vascular endothelial growth factor* and *matrix metalloproteinase-2* (44–46), or repressing metastasis suppressor genes, such as *RECK* (47)—is critical for the induction of cancer metastasis by transforming growth factor-β, AKT, and HER-2/*neu*. In contrast, Sp1 knockdown inhibits cancer metastasis. This supports our finding that exogenous COX-2 in lung cancer cells negatively regulates the expression of the invasion suppressor, CRMP-1, through activation of Sp1. It also implies that COX-2 expression may promote cancer metastasis through activation of Sp1, which, as indicated, not only inactivates anti-invasive genes such as *CRMP-1* but also activates metastasis-related genes (e.g., *matrix metalloproteinase-2*).

COX-2 inhibitors decrease Sp1 phosphorylation, Sp1-DNA binding, and vascular endothelial growth factor expression in pancreatic cancer (48). Sp1 mRNA levels were unchanged in colon cancer cells after treatment with a COX-2 inhibitor, but Sp1 protein was decreased by ubiquitin-dependent proteasomal degradation (49). By inducing the expression of the transcription factor EGR-1, which competes with Sp1 for overlapping DNA sequences, COX-2 inhibitors can also up-regulate the antitumorigenic protein, NAG-1 (50). We found that COX-2 inhibitors suppressed Sp1 in lung cancer cells, reducing Sp1-DNA binding at the promoter of *CRMP-1* and allowing the transcription factor, C/EBPα, to compete more successfully with Sp1.

In conclusion, our work suggests a causal link between COX-2 expression and down-regulation of the invasion suppressor, CRMP-1, in human lung cancer cells. COX-2 inhibitors can up-regulate CRMP-1 through reciprocal regulation of Sp1 and C/EBPα at the promoter region of the *CRMP-1* gene. Our study suggests that COX-2 inhibitors can inhibit cancer progression by activating an invasion suppressor, a pathway that represents a potential new target for the development of antimetastatic therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Yoshida BA, Sokoloff MM, Welch DR, Rinker-Schaeffer CW. Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl Cancer Inst 2000;92:1717–30.

2. Castelao JE, Bart RD III, DiPerna CA, Sievers EM, Bremner RM. Lung cancer and cyclooxygenase-2. Ann Thorac Surg 2003;76:1327–35.

3. Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. Cancer 1997;80:1529–37.

4. Steeg PS, Bevilacqua G, Kopper L, et al. Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst 1988;80:200–4.

5. Dong JT, Lamb PW, Rinker-Schaeffer CW, et al. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science 1995;268:884–6.

6. Lee JH, Miele ME, Hicks DJ, et al. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst 1996;88:1731–7.

7. Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res 2000;60:2764–9.

8. Chen JJ, Peck K, Hong TM, et al. Global analysis of gene expression in invasion by a lung cancer model. Cancer Res 2001;61:5223–30.

9. Shih JY, Yang SC, Hong TM, et al. Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells. J Natl Cancer Inst 2001;93:1392–400.

10. Shih JY, Lee YC, Yang SC, Hong TM, Huang CY, Yang PC. Collapsin response mediator protein-1: a novel invasion-suppressor gene. Clin Exp Metastasis 2003;20:69–76.

11. Wang LH, Strittmatter SM. A family of rat CRMP genes is differentially expressed in the nervous system. J Neurosci 1996;16:6197–207.

Published OnlineFirst June 4, 2008; DOI: 10.1158/1535-7163.MCT-08-0091

12. Su KY, Chien WL, Fu WM, et al. Mice deficient in collapsin response mediator protein-1 exhibit impaired LTP and impaired spatial learning and memory. J Neurosci 2007;27:2513 – 24.

13. Hamajima N, Matsuda K, Sakata S, Tamaki N, Sasaki M, Nonaka M. A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. Gene 1996;180:157 – 63.

14. Quinn CC, Gray GE, Hockfield S. A family of proteins implicated in axon guidance and outgrowth. J Neurobiol 1999;41:158 – 64.

15. Charrier E, Reibel S, Rogemond V, Aguera M, Thomasset N, Honnorat J. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. Mol Neurobiol 2003;28:51 – 64.

16. Arimura N, Inagaki N, Chihara K, et al. Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. J Biol Chem 2000;275: 23973 – 80.

17. Leung T, Ng Y, Cheong A, et al. p80 ROKα binding protein is a novel splice variant of CRMP-1 which associates with CRMP-2 and modulates RhoA-induced neuronal morphology. FEBS Lett 2002;532:445 – 9.

18. Khuri FR, Wu H, Lee JJ, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861 – 7.

19. Riedl K, Krysan K, Pold M, et al. Multifaceted roles of cyclooxygenase-2 in lung cancer. Drug Resist Updat 2004;7:169 – 84.

20. Masferrer JL, Leahy KM, Koki AT, et al. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 2000;60:1306 – 11.

21. Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J 2001;15: 2742 – 4.

22. Yao M, Lam EC, Kelly CR, Zhou W, Wolfe MM. Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. Br J Cancer 2004;90: 712 – 9.

23. Chu YW, Yang PC, Yang SC, et al. Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. Am J Respir Cell Mol Biol 1997;17:353 – 60.

24. Dohadwala M, Batra RK, Luo J, et al. Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion. J Biol Chem 2002;277:50828 – 33.

25. Gao J, Niwa K, Sun W, et al. Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and upregulate cyclooxygenase-2 protein expression in endometrial cancer cells. Cancer Sci 2004;95:901 – 7.

26. Brooks AR, Blackhart BD, Haubold K, Levy-Wilson B. Characterization of tissue-specific enhancer elements in the second intron of the human apolipoprotein B gene. J Biol Chem 1991;266:7848 – 59.

27. Su JL, Shih JY, Yen ML, et al. Cyclooxygenase-2 induces EP1- and HER-2/*neu*-dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Res 2004;64:554 – 64.

28. Tjiu JW, Liao YH, Lin SJ, et al. Cyclooxygenase-2 overexpression in human basal cell carcinoma cell line increases antiapoptosis, angiogenesis, and tumorigenesis. J Invest Dermatol 2006;126:1143 – 51.

29. Chang CC, Shih JY, Jeng YM, et al. Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. J Natl Cancer Inst 2004;96:364 – 75.

30. Bonello MR, Khachigian LM. Fibroblast growth factor-2 represses platelet-derived growth factor receptor-α (PDGFR-α) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical *cis*-acting element in the proximal PDGFR-α promoter. J Biol Chem 2004;279:2377 – 82.

31. Kavurma MM, Khachigian LM. Sp1 inhibits proliferation and induces apoptosis in vascular smooth muscle cells by repressing p21WAF1/Cip1 transcription and cyclin D1-4-p21WAF1/Cip1 complex formation. J Biol Chem 2003;278:32537 – 43.

32. Kavurma MM, Khachigian LM. Vascular smooth muscle cell-specific regulation of cyclin-dependent kinase inhibitor p21(WAF1/Cip1) transcription by Sp1 is mediated via distinct *cis*-acting positive and negative regulatory elements in the proximal p21(WAF1/Cip1) promoter. J Cell Biochem 2004;93:904 – 16.

33. Callejas NA, Bosca L, Williams CS, Du BR, Martin-Sanz P. Regulation of cyclooxygenase 2 expression in hepatocytes by CCAAT/enhancer-binding proteins. Gastroenterology 2000;119:493 – 501.

34. Halmos B, Huettner CS, Kocher O, Ferenczi K, Karp DD, Tenen DG. Down-regulation and antiproliferative role of C/EBPα in lung cancer. Cancer Res 2002;62:528 – 34.

35. Halmos B, Basseres DS, Monti S, et al. A transcriptional profiling study of CCAAT/enhancer binding protein targets identifies hepatocyte nuclear factor 3β as a novel tumor suppressor in lung cancer. Cancer Res 2004;64:4137 – 47.

36. Karlseder J, Rotheneder H, Wintersberger E. Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. Mol Cell Biol 1996;16:1659 – 67.

37. Khanna-Gupta A, Zibello T, Simkevich C, Rosmarin AG, Berliner N. Sp1 and C/EBP are necessary to activate the lactoferrin gene promoter during myeloid differentiation. Blood 2000;95:3734 – 41.

38. Tang QQ, Jiang MS, Lane MD. Repressive effect of Sp1 on the C/EBPα gene promoter: role in adipocyte differentiation. Mol Cell Biol 1999; 19:4855 – 65.

39. Kokawa A, Kondo H, Gotoda T, et al. Increased expression of cyclooxygenase-2 in human pancreatic neoplasms and potential for chemoprevention by cyclooxygenase inhibitors. Cancer 2001;91: 333 – 8.

40. Edwards J, Mukherjee R, Munro AF, Wells AC, Almushatat A, Bartlett JM. HER2 and COX2 expression in human prostate cancer. Eur J Cancer 2004;40:50 – 5.

41. Ranger GS, Thomas V, Jewell A, Mokbel K. Elevated cyclooxygenase-2 expression correlates with distant metastases in breast cancer. Anticancer Res 2004;24:2349 – 51.

42. Sinicrope FA, Gill S. Role of cyclooxygenase-2 in colorectal cancer. Cancer Metastasis Rev 2004;23:63 – 75.

43. Bradbury D, Clarke D, Seedhouse C, Corbett L, Stocks J, Knox A. Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites. J Biol Chem 2005;280: 29993 – 30000.

44. Pore N, Liu S, Shu HK, et al. Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism. Mol Biol Cell 2004;15:4841 – 53.

45. Bae IH, Park MJ, Yoon SH, et al. Bcl-w promotes gastric cancer cell invasion by inducing matrix metalloproteinase-2 expression via phosphoinositide 3-kinase, Akt, and Sp1. Cancer Res 2006;66:4991 – 5.

46. Jungert K, Buck A, von Wichert G, et al. Sp1 is required for transforming growth factor-β-induced mesenchymal transition and migration in pancreatic cancer cells. Cancer Res 2007;67:1563 – 70.

47. Hsu MC, Chang HC, Hung WC. HER-2/*neu* represses the metastasis suppressor RECK via ERK and Sp transcription factors to promote cell invasion. J Biol Chem 2006;281:4718 – 25.

48. Wei D, Wang L, He Y, Xiong HQ, Abbruzzese JL, Xie K. Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human pancreatic cancer via suppression of Sp1 transcription factor activity. Cancer Res 2004;64:2030 – 8.

49. Abdelrahim M, Safe S. Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins. Mol Pharmacol 2005;68: 317 – 29.

50. Baek SJ, Kim JS, Moore SM, Lee SH, Martinez J, Eling TE. Cyclooxygenase inhibitors induce the expression of the tumor suppressor gene EGR-1, which results in the up-regulation of NAG-1, an antitumorigenic protein. Mol Pharmacol 2005;67:356 – 64.

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Molecular Cancer Therapeutics

Modulation of the expression of the invasion-suppressor CRMP-1 by cyclooxygenase-2 inhibition via reciprocal regulation of Sp1 and C/EBPα

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