Transcriptional Co-activators CREB-binding Protein/p300 Increase Chondrocyte Cd-rap Gene Expression by Multiple Mechanisms Including Sequestration of the Repressor CCAAT/Enhancer-binding Protein*

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Cartilage-derived retinoic acid-sensitive protein (CD-RAP) is a small secreted matrix protein expressed in developing and adult cartilage and by chondrocytes in culture. We have previously shown that the expression of Cd-rap, like many other cartilage matrix proteins, is repressed by interleukin 1β and that the transcription factor CCAAT/enhancer-binding protein (C/EBP) β plays an important role in the interleukin 1β-induced repression (Okazaki, K., Li, J., Yu, H., Fukui, N., and Sandell, L. J. (2002) J. Biol. Chem. 277, 31526–31533). The co-activators CREB-binding protein (CBP) and p300 are transcriptional co-regulators that participate in the activities of many different transcription factors including C/EBP. Here we show that CBP/p300 can reverse the inhibitory effect of C/EBP and moreover can stimulate expression of Cd-rap. The mechanism of this effect is shown to involve a unique synergy whereby CBP/p300 stimulate Cd-rap gene expression by at least two mechanisms. First, binding of CBP/p300 to C/EBPβ leads to sequestration of C/EBP eliminating DNA binding and subsequent repression; second, binding of CBP/p300 to the transcriptional activator Sox9 increases Sox9 DNA binding to the Cd-rap promoter leading to further stimulation of gene transcription. This is an example of a complementary transcriptional network whereby two very different mechanisms act together to confer a functional increase in transcription. This new paradigm is likely generally applicable to cartilage genes as Col2a1 cartilage collagen gene responds similarly.

Cartilage-derived retinoic acid-sensitive protein (CD-RAP) is a small secreted matrix protein expressed in developing and adult cartilage and by chondrocytes in culture (1). It was originally cloned as an mRNA co-regulated with Col2a1 in chondrocytes and provides a reliable model for chondrocyte gene transcription (2). Melanoma inhibitory activity (MIA), the human homologue of Cd-rap, was isolated independently from a cell line derived from a brain metastasis of a human melanoma (3). Cd-rap/MIA is expressed by various tumor cells including melanoma, chondrosarcoma, and breast cancer, but its physiological expression is restricted primarily to cartilage (4). The protein structure of Cd-rap/MIA showed that it is a 56 kDa homologue 3 domain, a feature that is unique for an extracellular protein. We have shown previously that the expression of Cd-rap is repressed by interleukin 1β (IL-1β) (5), a major cytokine that mediates the inflammatory reaction, and is considered to play an important role in the cartilage degradation observed in osteoarthritis and rheumatoid arthritis. The transcription factor C/EBPβ plays an important role in the IL-1β-induced repression of both Cd-rap and Col2a1 (6).

C/EBPs are a family of transcription factors that contain a highly conserved, basic leucine zipper domain at the carboxyl terminus that is involved in dimerization and DNA binding. C/EBPβ mRNA can produce at least three isoforms by alternative initiation of translation, 38 kDa (liver-enriched activated protein (LAP-FL)), 35 kDa (LAP), and 20 kDa (liver-enriched inhibitory protein (LIP)), with the LAP and the LIP forms being the major polypeptides produced in cells (7). These two proteins share the 145 carboxyl-terminal amino acids that contain the basic DNA-binding domain and the leucine zipper dimerization helix (7) but differ at the amino terminus. IL-1β treatment of lung interstitial cells or rat chondrosarcoma (RCS) cells increases C/EBPβ mRNA and protein levels, including LAP and LIP (6, 8), and all forms of C/EBPβ and a related protein C/EBPδ repress Cd-rap gene expression. Col2a1 is also repressed by C/EBPβ and C/EBPδ (6).

Sox9, a high mobility group domain transcription factor, has been identified as a key regulator in chondrocyte differentiation (9). Sox9 is a member of a large family of proteins that harbor a DNA-binding domain with >50% similarity to that of sex-determining region Y, the testis-determining gene in mammals (10). Sox9 binds to and controls transcription factor of the Col2a1 (11) and Cd-rap (12) genes.

Co-activators CBP and p300 were originally identified as proteins that bind to the adenoviral E1A and the cAMP-response element-binding protein (CREB), respectively (10, 13). The CBP/p300 genes are conserved in a variety of multicellular organisms, from worms to humans, and are very similar in structure and function. The CBP/p300 proteins are very large transcriptional co-regulators that participate in the activities of many different transcription factors such as CREB, c-JUN,
and p53 including C/EBP. The simultaneous interaction of multiple transcription factors with CBP/p300 has been proposed to contribute to transcriptional synergy (14). p300 also enhances both basal Col1a2 promoter activity and transcription growth factor-β- or Smad3-induced activity (15). CBP/p300 control the transcription activity of cartilage homeoprotein-1 (cart1) through acetylation of a lysine residue that is highly conserved in other homeoproteins (16). CBP/p300 play pivotal roles in embryonic development and are implicated in tumorigenesis. The role of CBP/p300 in skeletal development and cancer was first suggested by the observations that the human CBP gene was disrupted in a dominant genetic disorder, Rubinstein-Taybi syndrome. Rubinstein-Taybi syndrome is characterized by craniofacial and limb defects and mental retardation as well as developmental anomalies of the eyes, heart, kidney, lung, skin, and testes (17). Rubinstein-Taybi syndrome results not only from gross chromosomal rearrangements of chromosome 16p but also from point mutations in the CBP gene itself (18). Because of this syndrome, we hypothesized that CBP/p300 could affect chondrocyte gene expression. To test this hypothesis, we investigated the effects of CBP/p300 on Cd-rap gene expression and the potential functional interaction of CBP/p300 with C/EBPβ. While this investigation was in progress, the interaction of the positive transcriptional regulator Sox9 with CBP/p300 was reported to affect Col2a1 gene expression (19). Consequently we broadened our studies to include Sox9. We found that CBP/p300 interact independently with Sox9 and C/EBPβ, functioning by different but complementary mechanisms to increase expression of cartilage genes. First we demonstrate a unique mechanism by which CBP/p300 bind to C/EBP and inhibit its DNA binding. Second CBP/p300 act to increase Sox9 binding to the Cd-rap promoter. Thus, removal of C/EBP repression by CBP/p300 occurs in concert with enhancement of Sox9-DNA binding to functionally increase transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Line**—The RCS cell line was grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum. This cell line was derived from a rat chondrosarcoma and provided by Dr. James H. Kimura. It is maintained in a 5% CO2 atmosphere at 37°C. All experiments were repeated a minimum of 3 times with 10% fetal calf serum. Northern Blot—Total RNA was isolated from culture cells using the RNeasy™ minikit (Qiagen, Valencia, CA). Ten micrograms of total RNA were separated on a 1.3% agarose gel and transferred to mixed ester nitrocellulose membrane (Amersham Biosciences). The membranes were prehydrated for 4 h and then hybridized as described before (6). The probes for CBP and p300 were generated by PCR as described previously (21).

**Antibodies**—Antibodies to C/EBPβ (C-19), C/EBPβ (C-22), Sox9 (P-20), and Ikaros (E-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to p300 (BW105) was purchased from Upstate Biotechnology (Lake Placid, NY).

**Plasmid Preparation**—The mouse Cd-rap promoter 5'-deletion constructs were made by PCR and subcloned into pGL3-basic vector as described previously (6). Site-directed mutagenesis within the Cd-rap promoter was performed by PCR (6). The following primers used in PCR were from Cd-rap promoter sequence: 5'-AGG-CTG ATT TGA ATT AAA TGT TGA GTA GGC TTC TAA ATT GGG CCA TTC AAA ACA TGA GAA CAA CAT GC-3' (sense for C/EBP MT in 2.2-kbp SOX MT Cd-rap) and 5'-GCA TGT TGT TCT CAT GTT TGT AAG AGT GCC CTA TAG ATT AAT ACA TGA CAA TG-3' (antisense for C/EBP MT in 2.2-kbp SOX MT Cd-rap). CMV-CEBPβ-FL, CMV-LAP, CMV-LIP, and CMV-CBPβ were prepared as described previously (6). CMV-Sox9 in pcDNA3 with amino-terminal FLAG epitope was a gift from Dr. Michael Wegner (22). CMV-p300 and CMV-CBP in pcDNA3 and serial deletions of GST-CBP were from Dr. Ralf Janknecht (23).

**Transient Transfection Assay**—RCS cells were transfected with FuGENE 6th transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Briefly 3 × 104 cells/well were cultured on a 6-well plate for 6 h. The transfection mixture contained 1–2.6 μl of FuGENE 6, 0.2–0.5 μg of promoter construct, 5–40 ng of Sox9, 30–400 ng of p300, 0.5–2.0 μg of C/EBPβ-FL, or 80 ng of pCMV-β-gal in a total volume of 100 μl. Due to low translation efficiency of C/EBPβ-FL, we used higher amounts of plasmid for transfection as shown previously (6). The relative rate of plasmid to promoter constructs was 1:4. The total amount of DNA per well was adjusted to 0.6 or 2.58 μg by the addition of vector alone. On the following day, medium was replaced, and cells were cultured for 36 h. CMV-C/EBPβ-FL, CMV-Sox9, CMV-CBP, and CMV-p300 were co-transfected with CMV-β-galactosidase as an internal control. The luciferase activities were assayed and normalized to β-galactosidase. Medium contained 10% fetal calf serum.

**Pull-down Assay**—Pull-down assays were performed using in vitro translated transcription factors and truncated GST fusion CMV-LAP, LIP, or Sox9 was translated in vitro with the TntT8 translation system (Promega, Madison, WI) according to the manufacturer’s instructions. To prepare radioactively labeled protein, each translation reaction contained 5 μCi of [35S]methionine (10 mCi/ml) (Amersham Biosciences) and mixture of amino acids and p300/lithium. A 1% volume of the supernatant was loaded on an SDS-polyacrylamide gel as the input fraction. GST fusion proteins were induced by isopropyl-1-thio-β-D-galactopyranoside. GST fusion proteins were mixed with 15 μl of gluthathione-Sepharose 4B (Amersham Biosciences) in 50% buffer slurry along with the remaining [35S]-labeled in vitro translated protein. For C/EBPβ, the mixture was incubated for 2 h at 4°C and washed three times with buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 0.5% Nonidet P-40, 0.5 mM phenylmethysulfonyl fluoride, and 1 mM dithiothreitol. For Sox9, the mixture was incubated with buffer containing 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 10% (v/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol.

**Co-immunoprecipitation Assay**—RCS cells grown in 100-mm tissue culture plates were treated for 48 h with 10 ng/ml IL-1β (R&D Systems, Minneapolis, MN). After a 48-h incubation with IL-1β, cells were washed twice with phosphate-buffered saline and lysed in Binding Buffer X after centrifugation at 800 g for 5 min at 4°C. Supernatants containing cellular lysates were incubated with an aliquoted 1 μg of protein in 500 μl, was incubated for 2 h at 4°C with 20 μl of antibody or anti-p300 antibody to increase Sox9 binding to the Cd-rap promoter. Ten nanograms of preimmune rabbit IgG, anti-C/EBPβ, or anti-p300 antibody were added to each tube and incubated overnight at 4°C. On the following day, 20 μl of protein A-agarose were added to each tube for 1 h at 4°C. The beads were washed twice with Binding Buffer X after centrifugation at 800 g for 5 min at 4°C. Immunoprecipitated samples and starting material were separated by SDS-PAGE (5–15% gradient gels) and blotted onto a Hybond-C Extra mixed ester nitrocellulose membrane (Amersham Biosciences). The membranes were immunoblotted with anti-C/EBPβ antibody or anti-p300 antibody and developed using an enhanced chemiluminescence substrate kit (Pierce). Two percent of input protein was resolved by SDS-PAGE.

**Electrophoretic Mobility Shift Assay (EMSA)**—Oligonucleotides were synthesized by Invitrogen, and complementary oligonucleotides were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. Oligonucleotide F3 spanning –2095 to –2063 bp of the Cd-rap promoter covered the C/EBPβ site (6). LIP, LAP, CBP, and p300 proteins were synthesized by in vitro translation. After purification of GST fusion proteins using gluthathione-Sepharose 4B and GST elution buffer (50 mMTris–HCl (pH 8.0), 20 mM reduced glutathione), the size and concentration of each fusion protein was verified by Coomassie Blue staining of the 10% SDS-polyacrylamide gel and the Bradford protein assay. The concentration of each was equalized with GST elution buffer. Reaction mixtures contained 2 μl of 5% EMSA buffer (25% glycerol), 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM MgCl2, 0.5 mM EDTA, 5 mM dithiothreitol), 2 μg of poly(dI-dC) with the 32P-labeled DNA probe, in vitro translated protein, antibodies as indicated, and water in a total volume of 10 μl. Binding reactions were incubated for 15 min at room temperature. Products were analyzed on 4% polyacrylamide gels (8 M urea, 0.1× Tris borate-EDTA buffer). The gels were dried and autoradiographed.

**Chromatin Immunoprecipitation (ChIP) Assay**—The ChIP assay was performed with a ChIP Assay kit (Upstate Biotechnology) according to the manufacturer’s instructions. Briefly 1.8 × 106 RCS cells were cultured on a 10-cm dish for 6 h. Then the RCS cells were treated with IL-1β (10 ng/ml). Two hours after the incubation with IL-1β, a transient transfection assay was performed. The transfection mixture contained

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isoforms of C/EBPβ and C/EBPβ also decreased the promoter activity (6). In contrast, co-transfection with either CMV-CBP or CMV-p300 strongly enhanced the Cd-rap promoter activity in a dose-dependent manner (Fig. 1A). To determine whether CBP/p300 could reverse the negative effect of C/EBPβ, transfection assays were performed with C/EBPβ-FL and p300 alone or together. As illustrated in Fig. 1B, co-transfection with p300 and C/EBPβ-FL together restored the Cd-rap promoter activity that was repressed by C/EBPβ-FL alone. CBP/p300 have also been shown to stimulate expression of chondrocyte-specific Col2a1 enhancer (19). Therefore, we hypothesize that CBP/p300 may enhance cartilage gene expression by a common mechanism.

C/EBPβ, but Not CBP/p300, Responds to IL-1β in RCS Cells—The previous experiment suggests that the ratio of C/EBP to CBP/p300 would have an effect on function (Fig. 1). We have shown that C/EBPβ protein and mRNA expression are increased upon stimulation with IL-1β (6) and that C/EBP proteins down-regulate Cd-rap gene expression. Therefore, we asked whether this ratio would change in the presence of IL-1β. To determine whether CBP/p300 respond to IL-1β, Western blot analysis was performed with RCS whole cell lysates. For C/EBPβ, after treating with IL-1β (10 ng/ml) for 48 h, the total amount of expression was increased, and one of the isoforms of C/EBPβ, LIP, was observed only after IL-1β treatment (Fig. 2A). For p300, the total amount of expression was not affected by IL-1β treatment. Quantification by densitometry showed 1.69 relative density for non-treatment and 1.74 relative density for IL-1β treatment (Fig. 2A). To investigate the expression of C/EBPβ, Sox9, and CBP/p300 genes, a Northern blot was performed. As shown in Fig. 2B, the mRNA level of C/EBPβ increased with time after treating with IL-1β; however, neither p300 nor CBP mRNAs changed over the 48-h time period (Fig. 2B). The mRNA level of Sox9 did not significantly change over the 48-h period assessed (Fig. 2B). Thus mRNA and protein results are consistent with a differential effect of IL-1β on C/EBPβ and CBP/p300 that potentially results in an increased ratio of C/EBP to p300 that would act to repress Cd-rap transcription.

C/EBPβ Interacts with a Specific Domain of CBP/p300 in RCS Cells—C/EBPβ has been shown to interact with p300 in the quail fibroblast cell line QT6 using overexpression of proteins (24, 25). To assess the endogenous intracellular association of C/EBPβ with p300 in chondrocytes, co-immunoprecipitation assays were performed in RCS cell lysates. RCS cells were treated with IL-1β (10 ng/ml) for 48 h to induce endogenous C/EBPβ. The cell lysate was incubated with antisera to C/EBPβ and p300 and preimmune IgG. Immunoblot analysis of the precipitated fraction with anti-C/EBPβ or anti-p300 showed that p300 co-precipitated with LAP in these cells (Fig. 3). C/EBPβ-FL and LAP cannot be distinguished on this Western blot. The isoform of C/EBPβ, LIP, was also observed to interact with p300 (Fig. 3, left panel). In Fig. 3, the immunocomplex with p300 contained more LIP than LAP. Two possibilities could account for this observation. First, p300 might have bound degraded C/EBP that reacts with anti-C/EBP antibody. Second, in IL-1β-treated RCS cells, LAP appears to be more abundant than LIP; however, LIP appears to bind more p300. Potentially these experiments indicate that LIP has a higher affinity for CBP/p300 (Fig. 3).

To further confirm this association between C/EBP isoforms and CBP/p300 and map the site of interaction, we performed a pull-down assay using the constructs consisting of small domains of CBP-GST fusion proteins (Fig. 4A). GST-CBP fusion proteins were synthesized and visualized by SDS-PAGE (Fig. 4B). The interaction between fragments of CBP and C/EBPβ

RESULTS

CBP/p300 Enhance the Cd-rap Promoter Activity and Overcome Repression by C/EBPβ—We have previously shown that the expression of the Cd-rap is down-regulated by IL-1β in bovine articular chondrocytes (5) and RCS cells (6). As a model for native chondrocytes, RCS cells were used to define the mechanism of IL-1β down-regulation of the Cd-rap mediated by the transcription factor C/EBP; the effect was then confirmed in human primary chondrocytes. RCS cells synthesize extracellular matrix in a manner very similar to that of chondrocytes. To test the effect of CBP/p300 on the Cd-rap promoter activity, a CBP/p300 expression plasmid was co-transfected into RCS cells with the 2.2-kbp Cd-rap promoter. As shown previously (6), co-transfection of CMV-C/EBPβ-FL decreased the Cd-rap promoter activity in a dose-dependent manner compared with the empty vector pcDNA3 (Fig. 1A).
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**Fig. 2.** Profile of protein expression of C/EBPβ and p300 in RCS cells treated with or without IL-1β. A, Western blot of whole cell lysates (left panel, 30 μg/lane; middle panel, 80 μg/lane; right panel, 30 μg/lane) extracted from RCS cells in the presence or absence of 10 ng/ml IL-1β for 48 h. Endogenous C/EBPβ or p300 was identified with anti-C/EBPβ antibody (left panel) or anti-p300 antibody (right panel). CRM, cross-reactive material. B, RCS cells were treated with 10 ng/ml IL-1β for various times as indicated. The levels of C/EBPβ, CBP, p300, Sox9, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by Northern blot (10 μg/lane). Asterisks indicate full-length GST fusion proteins. C, deletion mutants of GST-CBP fusion proteins were incubated with 35S-labeled LAP, LIP, or Sox9, and bound proteins were analyzed by autoradiography. Input protein is 2% of the total used. GST alone or deletion constructs Δ1–Δ6 are indicated.

**Fig. 3.** Co-immunoprecipitation of C/EBPβ with CBP/p300. One milligram of whole cell lysate was prepared from RCS cells treated with IL-1β (10 ng/ml) for 48 h and incubated with preimmune IgG, C/EBPβ antisemur, or p300 antisemur. The immune complexes were separated by electrophoresis and analyzed by immunoblotting with antibody to C/EBPβ (left panel) or p300 (right panel). ip, immunoprecipitate.

 Isoforms is shown in Fig. 4C. In vitro translated and radiolabeled LAP and LIP were used in these experiments. Autoradiography demonstrated that only the CH3 domain of CBP interacted with LAP and LIP but not GST alone. As a control, we confirmed that this domain of CBP also interacted with Sox9, recently reported by Tsuda et al. (19) (Fig. 4C). These data indicate that GST-CBP Δ5 that contains the CH3 domain of CBP was the only GST-CBP fusion protein that bound to C/EBPβ.

CBP/p300 Interferes with the DNA Binding Activity of C/EBPβ—To examine the effect of CBP/p300 on the DNA binding of C/EBPβ isoforms, EMSAs were performed. The oligonucleotide P3 is from −2095 to −2063 bp of the Cd-rap promoter and contains the C/EBP motif. A longer probe spanning −2121 to −2069 bp was also used with identical results (data not shown). Translated proteins used in these binding analyses were C/EBPβ and the truncated forms of C/EBPβ, LAP (missing 21 amino acids) and LIP (missing 151 amino acids). We have previously shown that C/EBPβ binds to this wild type probe but not to the mutant probe (6). C/EBPβ as well as C/EBPβ is known to be activated by IL-1β and tumor necrosis factor-α at the mRNA level (26) and the protein level (27). We have shown previously that LAP, LIP, and C/EBPβ were induced after IL-1β treatment in RCS cells (6). To confirm this binding was due to the C/EBP motif, we used the P3 Mut probe that contains mutations in the C/EBP motif. In EMSA with the P3 Mut probe, in vitro translated LIP, LAP, or C/EBPβ did not bind to this probe (data not shown). As shown in Fig. 1B, co-transfection with p300 and C/EBPβ-FL together restored the Cd-rap promoter activity that was repressed by C/EBPβ-FL alone, and we further showed direct interaction between C/EBP and CBP/p300 (Fig. 4C). Therefore, we hypothesized that CBP/p300 could alter the DNA binding activity of C/EBPβ. To test this hypothesis, we performed EMSAs to determine the effect of CBP/p300 on DNA binding activity of C/EBPβ. Oligonucleotide P3 was used as probe. First we tested whether in vitro translated CBP/p300 could bind to the probe DNA. CBP/p300 could not bind to the P3 probe under the conditions in
which LIP and LAP bound well (Fig. 5A). Next we determined the effect of CBP/p300 on DNA binding activity of in vitro translated C/EBPβ. Constant amounts of in vitro translated C/EBPs (LIP, LAP, and C/EBPβ) were added to the binding reaction along with increasing concentrations of in vitro translated CBP or p300. The results showed that the DNA binding activities of C/EBPβ and C/EBPδ were inhibited by both CBP and p300 in a dose-dependent manner (Fig. 5B). Identical results were observed with the longer oligonucleotide (data not shown).

As we showed in Fig. 4C, GST-CBP Δ5 was the only fusion protein that interacted with either C/EBPβ or Sox9. Therefore, we hypothesized that the CH3 domain of CBP would be an important domain that could alter the DNA binding of C/EBPβ or Sox9. To further examine the DNA binding activity of C/EBPβ, we used the constructs consisting of small domains of CBP, GST-CBP Δ1, Δ3, and Δ5, with in vitro translated LAP or LIP protein. GST-CBP Δ1, Δ3, and Δ5 alone did not bind to this P3 probe (Fig. 5C). GST-CBP Δ5 was the only fusion protein that affected the DNA binding of LAP and LIP (Fig. 5D). These results were consistent with the pull-down assays (Fig. 3C). These results also demonstrate that binding to CBP/p300 reduces the DNA binding of the C/EBPs, suggesting sequestration of the negative regulator from binding to DNA.

CBP/p300 Enhance the DNA Binding Activity of Sox9 to Cd-rap DNA—To determine the role of Sox9 in CBP/p300-mediated activation of Cd-rap transcription, Sox9 function was analyzed in a region of the Cd-rap gene known to bind Sox9 and to be important for function (12, 22). The oligonucleotide Cd-Rap (~412 to ~391 bp of the Cd-rap promoter) contains a dimeric SOX motif, and the oligonucleotide C/C mut is from the rat Protein zero promoter mutated to bind only one SOX protein (22) (Fig. 6A). As shown previously, Sox9 was able to form predominantly dimers with this Cd-Rap probe, and Sox9 formed predominantly monomers with the C/C mut (22) (Fig. 6B). With the addition of increasing amounts of p300 or CBP to the constant amount of Cd-Rap probe, binding to Sox9 was significantly enhanced and was in the dimeric form (Fig. 6C). This result is similar to the effect that was reported by Tsuda et al. (19) for the Col2a1 gene. We also examined whether the DNA binding activity of Sox9 was altered by a specific domain of CBP. Constructs synthesizing short domains of CBP were expressed using GST-CBP Δ1, Δ3, and Δ5. Alone, these proteins did not bind to the Cd-Rap probe (Fig. 6D). GST-CBP Δ5, encoding the CH3 domain, was the only fusion protein that altered the DNA binding of Sox9 (Fig. 6E). These results are also similar to the effect demonstrated by Tsuda et al. for the Col2a1 gene. In these experiments, the mobility of Sox9-DNA complexes was not altered by CBP/p300. This phenomenon is not unprecedented. Perhaps under our EMSA conditions, this interaction is unstable, and CBP/p300 may dissociate from the Sox9-DNA complex during electrophoresis. Another possibility is that an additional protein that can stabilize this ternary complex may exist (28). Additional experiments will be required to establish the mechanism of increased Sox9 DNA binding by CBP/p300.

![Fig. 5. CBP/p300 interfere with the Cd-rap DNA binding activity of C/EBPβ. A. EMSA was performed with a radiolabeled oligonucleotide encoded by −2095 to −2063 of the Cd-rap promoter. EMSA analysis of C/EBPβ, CBP, and p300 was performed to determine binding to the Cd-rap promoter DNA. LIP or LAP, CBP, and p300 were added to the P3 oligonucleotide. C/EBP or Sox9. To further examine the DNA binding activity of CBP/p300, a set of constructs containing small domains of CBP, GST-CBP Δ1, Δ3, and Δ5, were used. These results showed that the DNA binding activities of C/EBPβ and C/EBPδ were inhibited by both CBP and p300 in a dose-dependent manner (Fig. 5B). Identical results were observed with the longer oligonucleotide (data not shown).

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Previously shown that mutation at the removal of the SOX or C/EBP DNA binding sites. We have predicted that there is a functional interaction between Sox9 and C/EBP activity on the promoter. The results shown here would predict that there is a concentration and activity of both positive and negative regulatory factors. The rate of transcription is determined by the concentration of p300—

Removal of Functional C/EBP or SOX Site Alters the Effect of p300—The rate of transcription is determined by the concentration and activity of both positive and negative regulatory factors. The results shown here would predict that there is a functional interaction between Sox9 and C/EBP activity on the Cd-rap promoter in the presence of co-regulator p300. To test this prediction, promoter activity was determined after removal of the SOX or C/EBP DNA binding sites. We have previously shown that mutation at −410 (2.2 SOX MT Cd-rap) abrogates Sox9 stimulation of the 2.2-kbp Cd-rap construct (12). As shown in Fig. 7, increasing Sox9 concentration enhanced the Cd-rap gene expression with the 2.2-kbp promoter construct. As expected, mutation of this SOX site greatly reduced the stimulation of the Cd-rap by Sox9. The biphasic response to Sox9 observed here has been shown by Kypriotou et al. (29). The co-regulator p300 was able to enhance the expression of the control promoter construct to a greater extent than the Sox9-mutated construct due to the presence of the Sox9 protein binding site in the wild type promoter. As an indication that the effect of p300 could be influenced by the C/EBP binding site, a similar experiment was performed using the same control plasmid (2.2-kbp Cd-rap promoter) and the 2.0-kbp Cd-rap promoter in which the IL-1β-responsive C/EBP site was removed (Fig. 8A). After removing the IL-1β-responsive element from the 2.2-kbp Cd-rap promoter, some repression by C/EBP still remained (Fig. 8A). Without the C/EBP binding site, Sox9 and p300 were able to stimulate gene expression to a greater extend (Fig. 8B). Taken together these results show that the function of p300/CBP on the overall rate of gene expression acts through both the SOX and C/EBP sites.

Removal of Both C/EBP and SOX Sites Abrogates the Effect of p300—We have shown that Cd-rap gene activation by p300 is via interaction with C/EBP or Sox9 (Figs. 7 and 8). Therefore, we hypothesized that p300 will not enhance the Cd-rap promoter after mutation of both C/EBP and Sox9 sites. We constructed a double mutant in the 2.2 SOX MT Cd-rap that was mutated at both C/EBP and Sox9 sites (Fig. 8A). We compared the effect of p300 on the wild type 2.2-kbp Cd-rap promoter and the Sox9 site mutation in the Cd-rap promoter with the double mutant in the 2.2-kbp Cd-rap promoter (Fig. 8B). p300 was unable to alter gene expression without these sites (Fig. 8B). These results suggest that p300/CBP exert their effect through both C/EBP and SOX sites.

Chromatin DNA Binding of Endogenous C/EBPβ and Sox9 in Cd-rap Promoter Is Altered by Addition of p300—We have shown that the DNA binding of C/EBPβ or Sox9 was significantly altered by p300 in transient transfection assays (Fig. 1) and EMSA (Figs. 5 and 6). Therefore, we performed the ChIP assay to test the hypothesis that endogenous C/EBPβ and Sox9 binding to the Cd-rap promoter would be altered by addition of p300. Endogenous C/EBPβ protein binding to this promoter could not be observed (Fig. 10A, left panel, lane 2). After treatment with IL-1β (10 ng/ml) for 24 h, C/EBPβ bound to the IL-1β-responsive site (Fig. 10A, left panel). After co-transfection with p300, C/EBPβ binding was reduced by overexpression of p300 (Fig. 10A, left panel). Transfection reagent and transfection with vector alone did not affect the results (data not shown). In contrast, p300 enhanced the DNA binding of Sox9 (Fig. 10A, right panel). These results suggested that CBP/p300 enhance the Cd-rap promoter by removing the repressor.
C/EBP and also by enhancing the DNA binding of Sox9 in RCS cells.

**DISCUSSION**

Transcriptional co-activators CBP and p300 function as important elements in the transcription network in a number of ways: by linking individual transactivators via protein-protein interactions to the basal transcriptional machinery, by recruiting basal transcription factors (transcription factor IIB, TATA-box binding protein, and RNA polymerase II holoenzyme), by modifying chromatin structure via histone acetylation, and by recruiting other histone acetyltransferases such as steroid receptor co-activator-1 and p300/CREB-binding protein-associated factor (30). Here we show that CBP/p300 stimulate transcription by a novel mechanism. Our results suggest that CBP/p300 enhance the *Cd-rap* promoter by inhibiting the activity of repressor protein C/EBP/H9252. CBP/p300 inhibit C/EBP activity by the binding of C/EBP to its CH3 (E1A) domain and preventing C/EBP from binding to DNA, essentially lowering the effective concentration. This sequestration of the repressive factor in conjunction with enhancing DNA binding of the activator protein Sox9, shown recently by Asahara and colleagues (19) for the cartilaginous gene *Col2a1* and here for the *Cd-rap* gene, acts via a single set of co-regulators to enhance transcription. Chromatin immunoprecipitation assays confirm the changes in endogenous DNA binding induced by p300/CBP (Fig. 10). Thus the two mechanisms provide a cooperative transcriptional network designed to control cartilage gene transcription shown diagrammatically in Fig. 11. In this sense, the two mechanisms act similarly to the classic cooperative control mechanism of glycogen utilization whereby protein kinase A phosphorylation of glycogen synthetase inhibits the enzyme, whereas phosphorylation by the same enzyme stimulates phosphorylase, the
critical step in glycogen utilization (31).

Our results show that CBP/p300 inhibited the activity of C/EBP by preventing its binding to DNA, thus altering the concentration of active transcription factor. This is a novel control mechanism, support for which has only recently been published in another system (32). These recent studies address the mechanism of NF-kB suppression of the tumor suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10). NF-kB is released from IκB after treatment of cells with tumor necrosis factor or IL-1 and causes repression of PTEN gene expression. This suppression was independent of p65 DNA binding or transcription functions. The new mechanism involved sequestration of limiting pools of CBP/p300 by p65. In chondrocytes, Tan et al. (33) have shown that IL-1β represses Col2a1 expression partially mediated by early growth response-1. Co-transfection with CBP/p300 with C/EBP derepresses the DNA binding. E, CBP/p300 interact with C/EBP β and Sox9, inhibiting binding of C/EBP β and enhancing Sox9 binding.

Repression of transcription and the relief from repression are now recognized as important gene regulatory devices in chondrogenesis and a wide variety of other developmental and adult systems. The transcriptional repressor Nkx3.2 can activate the

**FIG. 9.** Mutation of both C/EBP and SOX sites abrogates the effect of p300. *A*, schematic of the luciferase construct of the C/EBP MT in 2.2-kbp SOX MT Cd-rap promoter in which both IL-1β-responsive C/EBP binding and SOX binding sites were inactivated. *B*, relative luciferase activities of RCS cells transfected with luciferase reporter containing 2.2-kbp Cd-rap promoter, 2.2-kbp SOX MT Cd-rap promoter in which only SOX binding was mutated, or C/EBP MT in 2.2 SOX MT Cd-rap promoter. Cells were transiently transfected with 0.2 μg of Cd-rap-luc; for vector alone, 0.4 μg; for p300, from left, 0.1 and 0.4 μg of expression plasmid; and for FuGENE 6, 1 μl were used. The absolute values of 2.2-kbp Cd-rap promoter, 2.2-kbp SOX MT Cd-rap promoter, and C/EBP MT in 2.2 SOX MT Cd-rap (both IL-1β-responsive C/EBP site and SOX site were mutated) were 1.04 × 10^6, 6.28 × 10^5, and 6.92 × 10^5 luciferase units, respectively. *Relative Luc. Activity* indicates fold expression relative to that seen with vector alone. Each bar represents the mean ± S.D. Cells were harvested 48 h after transfection.

**FIG. 10.** p300/CBP alter the effective concentration and binding affinity of C/EBP β and Sox9. *A*, ChiP assays were performed with RCS cells. Immunoprecipitations were performed with antibodies to C/EBP β, Sox9, and a control IgG. PCRs were carried out with the various ChiP samples, and the DNA control is DNA before immunoprecipitation. 0.1% of the total input DNA. *B*, schematic representation of primers in Cd-rap promoter region used for ChiP assay. ip, immunoprecipitate.

**FIG. 11.** A, C/EBP β and Sox9 bind to the Cd-rap promoter. B, interaction of CBP/p300 with Sox9 increases the DNA binding thus activating gene expression. C, C/EBP β alone represses transcription. D, interaction of CBP/p300 with C/EBP β derepresses the DNA binding. E, CBP/p300 interact with C/EBP β and Sox9, inhibiting binding of C/EBP β and enhancing Sox9 binding.
chondrocyte differentiation program in somatic mesoderm in a bone morphogenetic protein-dependent manner via a mechanism by which Sox9 transcription is stimulated by removal of a transcriptional repressor (1). We have shown that the transcriptional repressor AP-2 can inhibit chondrogenesis (35), and removal of the gene domain containing the C/EBP site used in this study derepresses transcription from the Cd-rap promoter in muscle, bone, epithelium, and liver. In addition four other repressive transcription factors have been reported in cartilage: nuclear factor of activated t cells transcription factor (36), Mxs2 (37), α,α crystallin-binding protein 1 (38), and δ-crystallin/E2-box factor 1 (39). In other gene systems, gene expression is induced by relief of repression: for example, serum-induced expression of cdc25A by relief of E2F-mediated repression (40, 41), CBP-induced expression of vitamin D receptor-mediated 2,5-hydroxylase by relief of YY1 repression (41), and AP-1-mediated relief of repression of CD30 (42). In a “fail safe” mechanism complementary regulation occurs in adenovirus type 12 tumorigenic cells whereby transcription shut off of major histocompatibility complex I genes is overcome by induction of NF-κB and relief of repression by the transcription factor chicken ovalbumin upstream promoter-transcription factor II (43)

Although our results demonstrate that CBP/p300 stimulate gene transcription by binding and sequestering the repressor protein C/EBPβ, we also show the additional stimulation by CBP/p300 that cannot be explained solely by inhibition of C/EBPβ. Therefore, we turned to the recent data presented by Tsuda et al. (19) showing that CBP/p300 also function as co-activators of Sox9 in chondrocyte differentiation. CBP/p300 enhance Sox9-dependent Col2a1 promoter activity, and disruption of the CBP-Sox9 complex (and potentially other CBP complexes) with a competing CB peptide during chondrogenesis in vitro inhibits Col2a1 mRNA expression. We have reported previously that Sox9 is able to bind to a SOX consensus sequence in the Cd-rap promoter, and mutation of the SOX motif led to decreased transcription of a Cd-rap promoter construct in chondrocytes (20). Here we show that mutation of SOX motif affects Cd-rap gene activation by p300 (Fig. 7). And we also show that mutation of C/EBP motif enhances Cd-rap gene activation by Sox9 or p300 (Fig. 8B). These results support the hypothesis that two mechanisms of the gene activation by p300 are utilized: interaction with Sox9 protein to enhance expression and interaction with C/EBP to inhibit repression (see Fig. 11).

In their effect on cartilage genes, CBP and p300 appear to function in the same way. Both CBP and p300 share several conserved regions, which constitute most of the known functional domains in the proteins. The domains are: the bromodomain, frequently found in mammalian histone acetyltransferases, three cysteine-histidine (CH)-rich domains (CH1, CH2, and CH3), a kinase-inducible interaction (KIX) domain, and an adenine deaminase 2 (ADA2) homology domain. The CH1, CH3, and KIX domains are considered to be important in mediating protein-protein interactions. The CH1 domain is also called transcriptional adapter zinc-binding (TAZ1) or CREB-binding, and the CH3 domain is also known as TAZ2 or E1A-binding. The CH3 domain was shown to interact with Sox9 (19) and with C/EBPβ (24). One of the CBP/p300 functions is to recruit RNA polymerase machinery. The CH3 domain is reported to be critical for recruiting RNA polymerase II or other basic transcriptional machinery (34). The general importance of the CBP/p300 CH3 domain was shown by Tsuda et al. (19) using a CH3 disrupter peptide to inhibit Col2a1 synthesis. This peptide disrupted the interaction with both Sox9 and CBP/p300 and C/EBP and CBP/p300. Although the CH3 domain is involved in Sox9 or C/EBPβ interaction, the CH1 domain may play a role in recruiting RNA polymerase II or other transcription factors in the cartilage transcriptional activation such as Col2a1 or Cd-rap. Because both Sox9 and C/EBP bind to the CH3 domain, there may be competition for binding if both factors are present. According to our in vitro binding assays (pull-down assays), interaction between CBP and either LAP or LIP was stable in 100 mM NaCl, although interaction between CBP and Sox9 was stable up to 500 mM NaCl. At 100 mM NaCl, Sox9 could also bind to the CH1 domain (data not shown). Interestingly this result helps to explain data presented by Tsuda et al. (19) where, although they did not observe binding of Sox9 to CH1 in their pull-down assays, removal of CH1 from p300 inhibited Col2a1 expression, indicating that CH1 was involved in the Sox9 effect on Col2a1 transcription. Consequently, under some conditions, the CH1 domain may play a role in binding of Sox9.

In summary, we demonstrated that CBP/p300 bind to endogenous C/EBP and increase Cd-rap gene expression by the novel mechanism of sequestering C/EBPβ, essentially inactivating the repressor. These results suggest CBP/p300 are important cofactors in chondrogenesis via down-regulation of C/EBPβ transcriptional activity. We have also shown that CBP/p300 have additional positive activity in the Cd-rap promoter due to the enhanced binding of Sox9 to the Cd-rap promoter as was shown recently for the Col2a1 promoter (19). Therefore two distinct but complementary mechanisms are active to increase gene transcription in chondrocytes via a cooperative network of transcriptional regulators and co-regulators. In light of these results, the skeletal phenotype of the CBP knock-out mice and the human disease Rubinstein-Taybi syndrome may be due to the lack of these specific activities of CBP during chondrogenesis leading to an abnormal endochondral bone formation and consequent skeletal abnormalities. In the same way, a change in the concentration of p300/CBP could have a significant effect on cartilage metabolism in joint diseases. This same cooperative network acts in the inflammatory response mediated by IL-1β. IL-1β induces C/EBP expression and repression of Sox9 in some cell types (20), working coordinately to down-regulate cartilage matrix genes. Although p300/CBP concentration remains the same in the presence of IL-1β (Fig. 2), the ratio of regulatory molecules is shifted toward down-regulation of cartilage genes, and binding to endogenous DNA is altered. When p300/CBP are increased, C/EBP can no longer bind to endogenous DNA, and Sox9 binds more tightly. Therefore, the ratio of p300/CBP to these two transcription factors can regulate gene expression. This may be an important mechanism in development and diseases where transcription rates of cartilage genes are known to be altered such as rheumatoid arthritis and osteoarthritis. The removal of gene repression via sequestration of inhibitory C/EBP by p300/CBP is a novel mechanism for enhancing transcription in chondrocytes.

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