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Critical role of C/EBPδ and C/EBPβ factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1β in articular chondrocytes

Béatrice Thomas[1], Francis Berenbaum[1], Lydie Humbert[1], Huimin Bian[2], Gilbert Béréziat[1], Leslie Crofford[2] and Jean Luc Olivier[1]

[1]*UPRES-A CNRS 7079, Université Pierre et Marie Curie, Paris, France;* [2]*Division of Rheumatology, University of Michigan, Ann Arbor, MI, USA*

The activity of the [−831; +103] promoter of the human cyclooxygenase-2 gene in cultured rabbit chondrocytes is stimulated 2.9 ± 0.3-fold by interleukin-1β and this stimulation depends on [−132; −124] C/EBP binding- and [−223; −214] NF-κB binding-sites. The C/EBPβ and C/EBPδ factors bind to the [−132; −124] sequence. The [−61; −53] sequence is also recognized by C/EBPβ and C/EBPδ as well as USF. Mutation of the whole [−61; −53] sequence abolished the stimulation of transcription but single mutations of the C/EBP or USF site did not alter the activity of the promoter, suggesting that the factors bound to the proximal [−61; −53] sequence interact with different members of the general transcription machinery. The [−223; −214] site binds only the p50/p50 homodimer and a non-rel-related protein, but not the transcriptionally active heterodimer p50/p65. The p50/p50 homodimer could interact with the C/EBP family members bound to the [−132; −124] sequence for full stimulation of the COX-2 transcription by interleukin-1β in chondrocytes. By contrast, the [−448; −449] sequence binds with a low affinity both the p50/p50 homodimeric and p50/p65 heterodimeric forms of NF-κB but has no role in the regulation of the human COX-2 promoter in chondrocytes.

Keywords: CAAT-enhancer-binding proteins; chondrocytes; cyclooxygenase-2; interleukin-1β; transcription.

Arthritic joints produce large quantities of prostaglandins, mainly prostaglandin E_2 (PGE_2). PGE_2 may be involved in the erosion of cartilage and juxtaarticular bone, and the induction of angiogenesis [1–3]. PG production is initiated by the two isoforms of cyclooxygenase, COX-1 and COX-2 (reviewed in [4,5]). These bifunctional enzymes convert arachidonic acid released from membranes by phospholipases A_2 [4] into PGG_2 (cyclooxygenase activity) and then PGG_2 into PGH_2 (hydroperoxidase activity). PGH_2 is converted into PGE_2 by a specific isomerase. COX-1 is a constitutive enzyme, while COX-2 gene expression is readily induced by inflammation [5].

Although IL-1α stabilizes COX-2 mRNA in human umbilical vein endothelial cells (HUVEC) [6], most work on the regulation of COX-2 gene expression emphasizes the role of several transcription factors, such as NF-κB and C/EBP family members. NF-κB acts on the human promoter at two putative sites, one at −448/−439 and the other at −223/−214 (Fig. 1A). The proximal site is involved in the stimulation of COX-2 gene transcription by hypoxia in HUVEC [7]. The mouse sequence homologous to the human distal site takes part in induction of the activity of the rodent promoter by TNFα in the osteogenic cell line MC3T3-E1 [8], and the binding of NF-κB to both the proximal and distal sites is correlated with the stimulation of COX-2 gene expression by IL-1β in human rheumatoid synoviocytes [9]. However, the mouse distal NF-κB site does not contribute to the induction of the promoter activity by LPS in RAW 264.7 macrophages [10] and mutation of the rat homologous sequence does not suppress the stimulation of the activity of the rat COX-2 promoter by TNFα in rat vascular smooth muscle cells [11], or the basal promoter activity in the pancreatic islet cell line HIT-T15 [12].

C/EBP factors are also involved in regulating the activity of the COX-2 promoter. There is a C/EBP binding site on the human promoter at positions −132/−124 downstream of an adjacent AP-2 site, which plays a role in the induction of the promoter activity by LPS in vascular endothelial cells [13]. The rat homolog of this C/EBP binding site is involved in cAMP-dependent regulation of COX-2 transcription by FSH and LH in rat granulosa cells [14], and in basal promoter activity in the pancreatic islet cell line HIT-T15 [12] and in the mouse skin [15]. The C/EBP family contains three main activating members, C/EBPα, C/EBPβ and C/EBPδ, which all recognize the same DNA sequence. C/EBPα is involved in hepatic and adipogenic differentiation, while C/EBPβ and C/EBPδ are responsible for the induction of several acute phase genes by IL-6 in the liver. Treating mice with LPS increases the transcription rate of the C/EBPβ and C/EBPδ genes in their kidneys and livers [16]. Phosphorylation of C/EBPβ by MAP kinases also increases the transactivating ability of this factor [17]. C/EBPα, C/EBPβ and C/EBPδ proteins have a common structure, with an N-terminal domain bearing the transactivation domain, a basic DNA-binding domain and a C-terminal

Correspondence to J. L. Olivier, UPRES-A CNRS 7079, Université Pierre et Marie Curie, 7 quai Saint Bernard, 75252 Paris Cedex 05, France. Fax: + 33 1 44275140, Tel.: + 33 1 44273256, E-mail: olivier@ccr.jussieu.fr

Abbreviations: C/EBP, CAAT enhancer binding proteins; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle's medium; IL-1β, interleukin-1β; LPS, lipopolysaccharide; PG, prostaglandin; $sPLA_2$-IIA, type IIA secreted phospholipase A_2; USF, upstream stimulatory factors.

Enzymes: Cyclooxygenase-2 (prostaglandin–endoperoxidase synthase 2, EC 1.14.99.1; Swiss-Prot accession number P35354); secreted phospholipase A_2 type IIA (PA2M_human, EC 3.1.1.4; Swiss-Prot accession number P14555).

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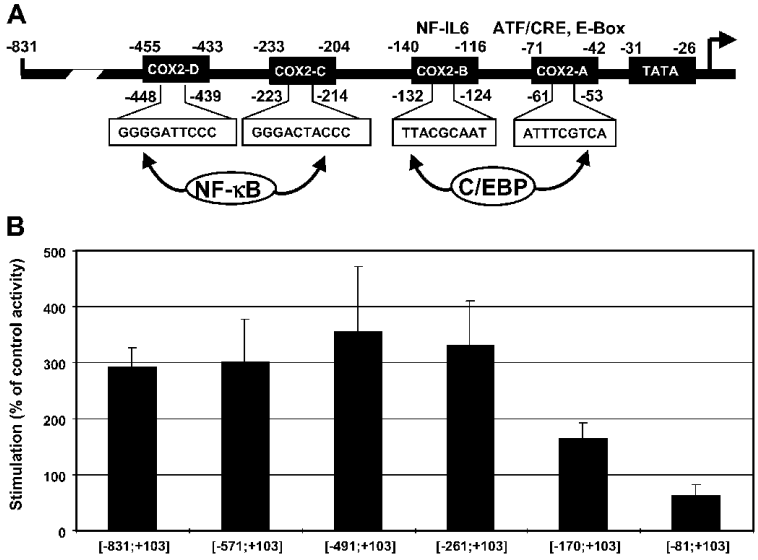


Fig. 1. IL−1β stimulation of the transcription activities induced by various 5′ deleted fragments of the human COX-2 promoter in primary cultures of rabbit chondrocytes. (A) Locations of the putative NF-κB and C/EBP binding sites on the human COX-2 promoter. The sequences of the putative binding sites are indicated in frames as are their locations on the promoter and the locations of the COX2-A, COX2-B, COX2-C and COX2-D oligonucleotides. The regulatory proximal element COX2-A is also described as a ATF/CRE and E-box like sequence. The sense strand of the [−132; −124] sequence (COX2-B) fits with the C/EBP consensus site 5′-TT/GNNGNAAT-3′. By contrast the [−61; −53] sequence (COX2-A) is homologous to the C/EBP consensus site in the antisense orientation. (B) Chondrocytes at 60% confluence were transfected by the calcium phosphate coprecipitation method using 12 μg of the various CAT constructs, 2.5 μg of the CMV-β-gal expression vector; 24 h after transfection, the cells were incubated with or without IL-1β (10 ng·mL^{-1}) for an additional 24 h and then harvested for measurement of CAT and β-galactosidase activities. The percentages of stimulation by IL-1β were calculated relative to the transcription activity obtained for each construct without IL-1β. Results are expressed as the means ± SEM of four independent transfections performed in duplicate.

domain containing a leucine zipper, which allows the homo- or heterodimerization of these factors [18].

Finally, the activities of the rodent and human COX-2 promoters are regulated by a proximal element that is located 20 bp upstream of the TATA box, but there are conflicting data on the role and the nature of the proteins bound to this promoter [10–14,19–23]. This element contains a perfect E-box 5′-CACGTG-3′ in the mouse and rat promoters, but the homologous sequence on the human promoter differs by one base pair [19] (Fig. 1). An ATF/CRE 5′-CGTCAC-3′ overlaps the E-box in the murine and human promoters [20,21]. Different factors therefore bind to this proximal element, depending on the species and on the cell. ATF and CREB family members bind to the murine sequence in NIH3T3 cells, but the response to mitogenic stimuli in these cells is mediated by c-jun, which heterodimerizes with the ATF family members [22,23]. The rat COX-2 promoter is also not recognized by ATF/CRE activities in ovarian granulosa cells, but the E-box binds USF factors and is required for basal transcription and its stimulation by hormones [19]. The ATF/CRE sequence is also required for basal expression of the human COX-2 gene in the macrophage cell line U937 [21] and actually binds C/EBPδ instead of ATF/CREB family members in vascular endothelial cells transfected by C/EBP expression vectors [13]. Both this sequence and the distal −132/−124 C/EBP binding site are required for the human COX-2 promoter to be induced by LPS in these cells [13]. In contrast, the E-box is not required for induction of the promoter activity by LPS in RAW 264.7 macrophages while mutation of the CRE sequence drastically reduces both the basal and stimulated transcription activities [10].

IL-1β causes expression of both the type IIA secreted phospholipase A_2 (sPLA$_2$-IIA) [25] and the COX-2 genes [24] in chondrocytes. Arachidonic acid may be provided to COX-2 by sPLA$_2$-IIA, and we have demonstrated that the binding of sPLA$_2$-IIA to chondrocyte membranes correlates with increased PGE_2 production [24]. COX-2 and sPLA$_2$-IIA are therefore functionally linked. We have also shown that C/EBPδ plays a critical role in the activation of the sPLA$_2$-IIA promoter by IL-1β in primary cultures of rabbit chondrocytes [25]. The present study was undertaken to determine whether both sPLA$_2$-IIA and the COX-2 gene can be induced by the same transcription factors in response to IL-1β and whether these factors may be putative therapeutic targets at which to block inflammation. We examined the roles of the C/EBP family members and their collaboration with c-rel family members in the regulation of COX-2 gene transcription in chondrocytes. We also characterized other transcription factors, specifically the USF proteins, that recognize regulatory elements in the human COX-2 promoter and determined the contributions of each of these cis-elements to IL-1β-stimulated transcription.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 kinase, ligase and Taq polymerase were purchased from Biolabs (USA). Oligonucleotides were provided by Oligoexpress (France). Materials for cell culture and protein molecular weight markers were from Sigma (USA) (Dulbecco's modified Eagle's medium, HAM's F12 medium, fetal bovine serum, Hepes, trypsin, glutamine, penicillin–streptomycin), Gibco BRL (Gey medium) and Costar (USA) (flasks and Petri dishes). The β-galactosidase expression vector CMVβ-gal was obtained from Clontech (USA), and poly(dI·dC) and deoxynucleotides from Pharmacia (Sweden). Radioactive

products were supplied by Amersham (UK). Collagenase, hyaluronidase and trypsin for preparing monolayers of rabbit chondrocytes were supplied by Boehringer Mannheim (France). The antibodies against the NK-κB subunits p50 and p65 and the C/EBP family members C/EBPα, C/EBPβ and C/EBPδ were provided by Santacruz Biotechnology (USA). The antibodies to USF1 and USF2 were gifts from M. Raymondjean (ICGM Cochin, Paris, France).

Chondrocyte cultures and transfections

Female 3-week-old Fauve de Bourgogne rabbits were killed and the shoulders, knees and femoral heads were dissected out under sterile conditions [26]. The articular cartilage was removed, cut into small pieces, and digested at 37 °C with 0.05% hyaluronidase in Gey medium for 15 min, then with 0.25% trypsin for 30 min, and lastly with 0.2% collagenase for 90 min. The resulting chondrocytes were washed in HAM's F12 medium without fetal bovine serum for 60 min, suspended in HAM's F12 medium supplemented with 10% fetal bovine serum and seeded into 60 mm dishes (1.5×10^5 cells per dish). The cells were maintained at 37 °C in 5% CO_2 and the culture medium was changed every 2–3 days. The cells reached preconfluency within 6–7 days.

Chondrocytes were transfected using the calcium phosphate DNA coprecipitation method [27]. Cells were placed in DMEM and incubated with the transfection mixture containing 12 μg pUC-SH-CAT constructs and 2.5 μg of plasmids bearing the β-galactosidase gene, for 4 h. They were then shocked with HBS buffer (21 mM Hepes, pH 7.1, 16 mM dextrose, 0.8 mM NA_2HPO_4, 5 mM KCl, and 137 mM NaCl) containing 15% glycerol for 90 s. The treated cells were incubated for 20 h in HAM's F12 without fetal bovine serum and grown for an additional 24 h in HAM's F12 without fetal bovine serum, with or without IL-1β (10 $ng \cdot mL^{-1}$). The harvested cells were lysed by incubation with 50 μL 100 mM Tris pH 7.8, 0.7% NP40 for 15 min at 4 °C. CAT activities were measured by the two-liquid phases method [28]. β-Galactosidase activities were measured to normalize variations in transfection efficiency. Transfection experiments were performed in duplicate and repeated four times with two different preparations of plasmids.

Plasmid constructions

The various 5′ deleted fragments of the human COX-2 promoter from the −831, −571, −491, −261, −170, −81 to the +103 positions were amplified by PCR and inserted into the pCATBASIC plasmid (Promega, USA) upstream of the CAT gene. The [−223; −214] 5′-GGGACTACCC-3′ proximal putative NF-κB site on element COX2-C was mutated into the 5′-GGGACT**CGAG**-3′ sequence in the [−261; +103]-Cmut construct. This mutation introduced an additional *Xho*I site. The [−132; −124] 5′-GGGCTTACGCAAT-3′ putative distal C/EBP binding site on the COX2-B element was replaced by the GGG**GTGAATTCG**AT-3′ sequence, which contains an *Eco*RI site, and the mutant promoter was cloned into the [−261; +103]-Bmut CAT construct. The whole [−62; −50] 5′-CATTTCGTCACAT-3′ sequence of the COX2-A element was mutated into the 5′GAATTCGAGCTCG-3′ in the [−261; +103]-Amut1 CAT plasmid, whereas the partially mutated 5′-CA**ACG**CGTCACAT-3′ and 5′-CATTTCGTCA**GC**T-3′ sequences were inserted into the [−261; +103]-Amut2 and [−261; +103]-Amut3 CAT plasmids.

The PHD expression vectors containing the C/EBPβ and C/EBPδ cDNAs were a gift from G. Ciliberto (IRBM, Rome, Italy). The expression vector of the undegradable mutant of I-κB was generously given by A. Israël (Paris, France).

Preparation of nuclear extracts and cell lysates

Nuclear extracts of chondrocytes were prepared as previously described [29]. Briefly, confluent cells from three P100 dishes were grown in HAM's F12 without fetal bovine serum and then incubated in HAM's F12 with or without IL-1β (10 $ng \cdot mL^{-1}$) for 24 h. They were then washed and scraped off in NaCl/P_i. The cells were centrifuged at 1500 ***g*** for 5 min and the pellet was suspended in 500 μL buffer A (5 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP40, 0.5 mM dithiothreitol, 0.1 mM phenylmethanesulfonylfluoride, 5 $\mu g \cdot mL^{-1}$ leupeptin, 50 mM NaF). The cells were incubated at 4 °C for 15 min, centrifuged at 6000 ***g*** for 10 min, and the pellet suspended in 100 μL buffer C (20 mM Hepes pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride, 5 $\mu g \cdot mL^{-1}$ leupeptin, 50 mM NaF). The nuclei were lysed by pipetting up and down four times and incubating for 30 min at 4 °C. The lysates were centrifuged at 100 000 ***g*** for 30 min at 4 °C in a TLC centrifuge (Beckman, USA). The supernatants were collected and the protein concentrations were measured according to Kalb & Berlohr [30]. The nuclear protein batches were stored at −80 °C. Lysates of COS-1 cells were prepared 40 h after transfection of the cells with the C/EBP expression vectors according to Olivier *et al.* [31].

Bandshift assays

The COX2-D (5′-CGGGAGAGGGGATTCCCTGCGCC-3′), COX2-C (5′-CAGGAGAGTGGGGACTACCCCCTCTGCTCC-3′), COX2-B (5′-CACCGGGCTTACGCAATTTTTTTAA-3′), COX2-A (5′-AGAAACAGTCATTTCGTCACATGGGCTTGG-3′) oligonucleotides corresponding to the regulatory elements of the human COX-2 promoter and the KBEwt oligonucleotide (5′-ACAGAGGGGACTTTCCGAGAG-3′) corresponding to the enhancer of the light chain κ gene [32] were used as probes. These double-stranded oligonucleotides (100 ng) were labeled using T4 kinase and 50 μCi [γ-^{32}P] ATP. Free nucleotides were separated from the labeled probe on a Sephadex G50 column. The specific activity of the probe was estimated by spotting 1 μL of the labeling mixture (before the G50 column) onto a TLC plate, separating the labelled probe and free nucleotide by chromatography and counting them. The specific activities were $1–2 \times 10^8$ $cpm \cdot \mu g^{-1}$. Chondrocyte nuclear extracts (6–9 μg) were incubated at 4 °C for 15 min in a 20-μL reaction solution containing 25 mM Hepes pH 7.6, 8% Ficoll, 40 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 3 μg of double-stranded poly(dI·dC). When double-stranded competitor oligonucleotides were used, they were added in a volume of 1 μL and the reaction mixture was incubated with the nuclear extracts at 4 °C for 15 min before adding the probe. The competitors used in bandshift assays were the D-Alb oligonucleotide 5′-TGGTATGATTTTGTAATGGGGTAGGA-3′, which corresponds to the element D of the murine albumin promoter and binds C/EBP proteins [33], and the USF oligonucleotide 5′-TGGTCACGTGGCCTACACCTATAA-3′, which corresponds to the enhancer of the murine immunoglobin heavy chain gene and binds USF proteins [34]. The sequences of the other oligonucleotides used as competitors are indicated in the corresponding figures. Double-stranded oligonucleotide probes (100 000 cpm) were then added and the incubation was continued for 30 min at 4 °C. Free DNA and DNA–protein complexes were resolved by

electrophoresis in 5% or 7% polyacrylamide gels in 6.7 mM Tris/HCl, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9. The gels were dried and exposed to X-OMAT® film (Kodak, USA). In supershift experiments, chondrocyte nuclear extracts were preincubated for 15 min at 4 °C with 1 μL of specific antibodies.

RESULTS

Requirement of the [−261; −81] fragment of the human COX-2 promoter for stimulation of its activity by IL-1β in chondrocytes

The human COX-2 promoter contains two putative binding sites for NF-κB and the C/EBP factors (Fig. 1A). The proximal putative C/EBP binding sequence is also a putative ATF/CRE site overlapping an E-box-like sequence. We named the elements containing the proximal CRE and the distal C/EBP binding sites COX2-A and COX2-B, respectively, and the elements enclosing the putative NF-κB sites COX2-C and COX2-D (Fig. 1A). Primary cultures of rabbit chondrocytes were transiently transfected by CAT constructs containing 5′-deleted fragments of the COX-2 promoter to determine the regions responsible for stimulation by IL-1β. Incubating the cells with IL-1β for 24 h increased the activity of the [−831; +103] fragment of the COX-2 promoter 2.9 ± 0.3-fold (Fig. 1B). The stimulation of the promoter activity by IL-1β was not modified by deletions down to the −261 position; the last deletion removed the putative distal −448/−439 NF-κB binding site (Fig. 1A,B). By contrast, deletion of the [−261; −170] fragment, containing the proximal NF-κB −223/−214 putative binding site, reduced the stimulation of the transcription activity by IL-1β to 1.6 ± 0.3-fold, and deletion of the [−170; −81] fragment, which suppressed the distal C/EBP binding sites but preserved the CRE, abolished all induction of transcription by IL-1β (Fig. 1B). This result indicates that the [−170; −81] fragment, which contains the [−140; −116] COX2-B element, is critical for the regulation of the human COX-2 promoter by IL-1β in chondrocytes and suggests that the C/EBP factors are involved in this regulation. In addition, deletion of the [−261; −170] fragment increased the basal activity of the COX-2 promoter 2.6 ± 0.3-fold (data not shown).

Need for binding of C/EBPβ and C/EBPδ to the [−140; −116] COX-2B element of the human COX-2 promoter for stimulation of its activity by IL-1β in primary cultures of rabbit chondrocytes

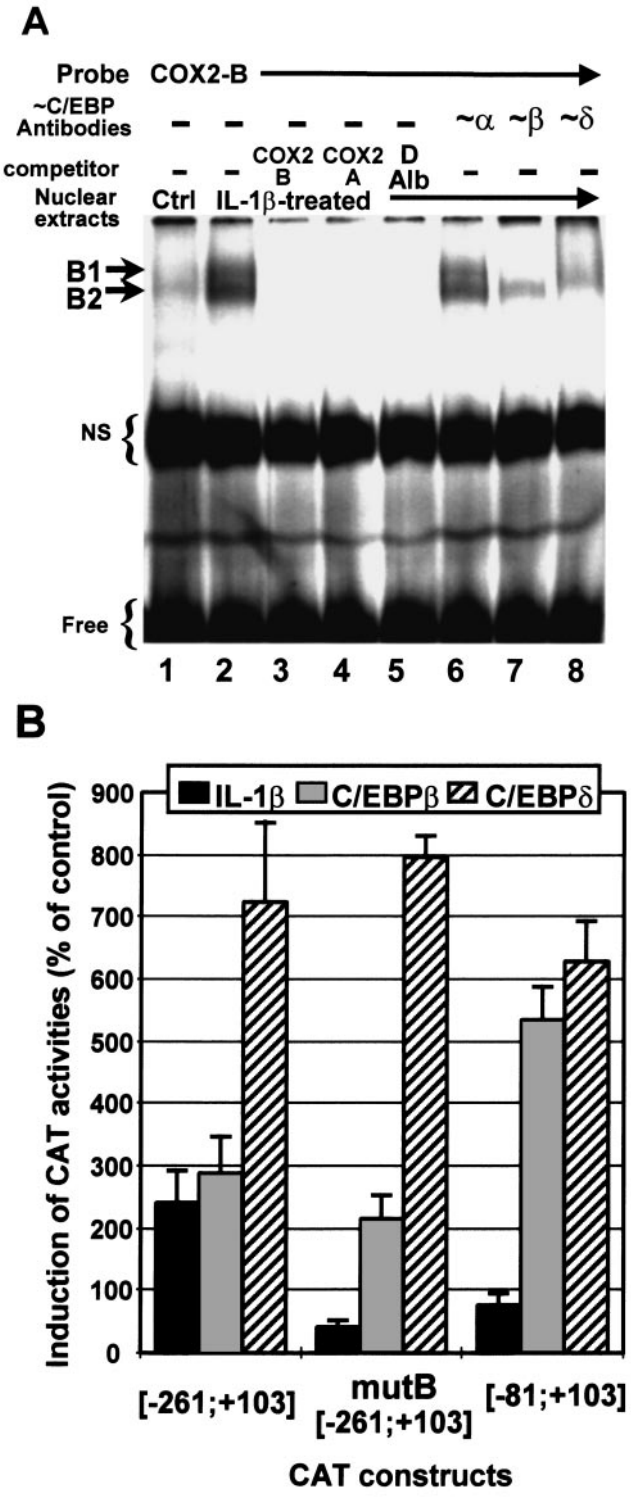
The oligonucleotide corresponding to the [−140; −116] COX2-B element formed two complexes with the chondrocytes nuclear extracts, B1 and B2, whose intensities were increased when the cells were first treated with IL-1β (Fig. 2A, compare lanes 1 and 2). Both these complexes were competed out by 100-fold excesses of the unlabeled COX2-B, COX2-A and D-Alb oligonucleotides. The D-Alb oligonucleotide corresponds to the D enhancer of the murine albumin promoter, which binds C/EBP factors [33] (Fig. 2A, lanes 3–5). This result confirms that the COX2-B and COX-2A elements bind C/EBP family members. An antibody against C/EBPα did not alter any of the B1 and B2 complexes (Fig. 2A, lane 6). Because all the C/EBP family members have the same binding site, rabbit articular chondrocytes probably do not produce C/EBPα. Antibodies to C/EBPβ and C/EBPδ totally supershifted the B1 complex (Fig. 2A, lanes 7, 8). By contrast the B2 complex was only partially displaced by both these antibodies (Fig. 2A, lanes 7 and 8). Considering that C/EBPβ and C/EBPδ bind to DNA by forming heterodimers as well as homodimers, these data indicate that the B1 complex is formed by C/EBPβ–C/EBPδ heterodimers, while the B2 complex is heterogeneous and corresponds to comigrating C/EBPβ–C/EBPβ and C/EBPδ–C/EBPδ homodimers.

Mutation of the [−132; −124] C/EBP binding sequence in a CAT construct containing the [−261; +103] fragment of the COX-2 promoter suppressed the stimulation of the transcription activity by IL-1β in primary culture chondrocytes (Fig. 2B). Cotransfections of C/EBPβ and C/EBPδ expression vectors led to enhancement of the activity of the wild type [−261; +103] promoter by 2.9 ± 0.6 and 7.2 ± 1.3-fold, respectively. Mutation of the [−132; −124] C/EBP binding site in the COX2-B element did not affect the stimulation of the promoter activity by C/EBPβ or C/EBPδ. However, as this mutation decreased the basal promoter activity twofold, the transactivation of the mutant promoter by C/EBPβ and C/EBPδ was also reduced twofold (data not shown). Furthermore, the [−81; +103] fragment of the promoter, which contains only the COX2-A element and the TATA box, is still transactivated by C/EBPβ and C/EBPδ (Fig. 2B). These results indicate that C/EBPβ and C/EBPδ must bind to the COX2-B element for the transcription of the human COX-2 gene to be stimulated by Il-1β in primary cultures of chondrocytes; they also suggest that C/EBPβ and C/EBPδ have alternative effects that might be mediated through their binding to the COX2-A element.

Need for binding of USF and C/EBP family members to the [−71; −42] COX2-A element for the activity of the human COX-2 promoter in primary cultures of rabbit chondrocytes

The COX2-A element formed three complexes with the chondrocyte nuclear extracts, A1, A2 and A3 (Fig. 3B, lane 1). The A1 and A2 complexes migrated very close together and were barely resolved. There was also a smeared band migrating just below the A1 complex and superimposed on the A2 complex. The intensities of the A1, A2 and A3 complexes were increased when the cells were treated with IL-1β prior to extraction of the nuclear proteins (Fig. 3B, compare lanes 1 and 2). The A2 and A3 complexes were competed out by a 100-fold excess of the unlabeled C/EBP binding oligonucleotide D-Alb (Fig. 3C, lane 2). The A2 and A3 complexes were supershifted by antibodies against C/EBPβ and C/EBPδ, but not by antibodies to C/EBPα (Fig. 3C, lanes 3–6), similarly to the B1 and B2 complexes formed with the COX2-B element. This indicates that these complexes corresponded to homodimers or heterodimers of C/EBPβ and C/EBPδ, like the B1 and B2 complexes. The formation of the A1 complex was prevented by adding a 100-fold excess of the USF binding oligonucleotide and the antibody to USF1/2 (Fig. 3C, lanes 6 and 7). The [−62; −50] 5′-CATTTCGTCACAT-3′ sequence must be responsible for the formation of the A1 and A3 complexes, because their formation was not impeded by adding a 100-fold excess of the unlabeled oligonucleotide Am1, in which this sequence was mutated (Fig. 3A and Fig. 3C, lane 8). Two oligonucleotides, Am2 and Am3, which contain mutations of the −60/−58 and −52/−51 nucleotides, respectively, were also used as competitors to localize the C/EBP and USF binding sites within the [−62; −50] sequence (Fig. 3A,D). The A2 and A3 complexes were competed out by the Am3 oligonucleotide, but not by Am2 (Fig. 3D, lanes 1 and 2). By contrast, the complex A1 was competed out by the Am2 oligonucleotide, but not by Am3 (Fig. 3D, lanes 1 and 2). Finally, the smeared band migrating

just below the A1 complex was not suppressed by 100-fold excesses of C/EBP-binding or Am3 competitors (Fig. 3C and Fig. 3D, lanes 2). These results indicate that the −60/−58 nucleotides are critical for the binding of C/EBP family members, which generate the A2 and A3 complexes. These nucleotides are located just upstream of the putative ATF/CRE sequence 5′-CGTCAC-3′ (Fig. 3A). They also show that the [−56; −51] 5′-GTCACA-3′ E-box-like sequence, which is altered in the Am3 oligonucleotide, is involved in the binding of USF.



Mutation of the whole [−62; −50] sequence in the COX2-A element suppressed the stimulation of the [−261; +103] promoter activity by IL-1β. It also suppressed the stimulation of the promoter activity by exogenous C/EBPβ and greatly reduced that by C/EBPδ (Fig. 4). Surprisingly, individual mutations of the USF or C/EBP binding sites similar to those performed in the Am2 and Am3 oligonucleotides did not alter the induction by IL-1β. Transactivations by exogenous C/EBPβ and C/EBPδ were slightly decreased by the Am2 mutation but unaffected by the Am3 mutation (Fig. 4). Finally, the Am1, Am2 and Am3 mutations did not significantly modify the basal activity of the [−261; +103] promoter (Fig. 4). Although C/EBPβ and C/EBPδ bind to the COX2-A as well as to the COX2-B elements, these results indicate that their binding to these two elements have different consequences. The binding of C/EBPβ and C/EBPδ to the COX2-B element is critical for stimulation of the promoter activity by IL-1β, while C/EBP factors can be replaced by USF on the COX2-A elements to induce COX-2 gene transcription in chondrocytes. This aspect of the regulation of the COX-2 gene transcription is further discussed below.

NF-κB binds to the −448/−439 and −223/−214 sequences of the human COX-2 promoter but the −223/−224 sequence is involved in the regulation of transcription by IL-1β in chondrocytes

We identified the contribution of NF-κB to the stimulation of the activity of the human COX-2 promoter in chondrocytes by determining whether this factor actually bound to the putative proximal and distal sites. NF-κB is formed by the association of two subunits, p50 and p65. The p65 subunit supports a strong transactivation ability but has a poor affinity for DNA, while p50 binds strongly to DNA but is poorly active [35]. NF-κB is translocated to the chondrocyte nuclei during the first hour of

Fig. 2. The [−140; −116] COX2-B element binds C/EBPβ and C/EBPδ factors and is critical for stimulation of COX-2 promoter activity by IL-1β in chondrocytes. (A) The 5′ end ^{32}P labeled COX2-B probe (100 000 cpm) were incubated with 3 μg poly(dI-dC) and 6 μg nuclear extracts of untreated- (lane 1) or IL-1β treated chondrocytes (lanes 2–8). Primary cultures of rabbit chondrocytes were incubated with 10 ng·mL^{-1} IL-1β for 24 h prior to extraction of nuclear proteins. 100-fold excesses of unlabeled COX2-B, COX2-A, and D-Alb oligonucleotides over the COX2-B concentration were incubated with the nuclear extracts for 15 min before adding the probe (lanes 3–5). The D-alb oligonucleotide corresponds to the regulatory element D of the murine albumin promoter which binds the C/EBP family members. Antibodies (1 μL pure commercial solution, Santa Cruz Inc, USA) against C/EBPα (lane 6) C/EBPβ (lane 7) or C/EBPδ (lane 8) were incubated with chondrocyte nuclear extracts for 15 min at 4 °C prior to adding poly(dI·dC). NS indicates a nonspecific band. B1 and B2 complexes are indicated by arrows. Electrophoresis was run on a 7% 30 : 1 bisacrylamide/acrylamide gel, which was dried and autoradiographed overnight. (B) Rabbit chondrocytes were transfected as indicated in Fig. 1 with the CAT constructs containing the wild-type [−261; +103] promoter, its mutant homolog for element B or the wild-type [−81; +103] promoter. The mutations of the COX2-B elements are indicated in the Materials and methods section. Cells were cultivated with or without IL-1β (10 ng·mL^{-1}) as indicated in Fig. 1. The CAT construct were cotransfected with 1 μg of the PHD expression vectors containing C/EBPβ (dotted bars) or C/EBPδ (dashed bars) cDNAs. Stimulations of the CAT activity were calculated relatively to that induced by the wild type or mutant [−261; +103] CAT constructs in the absence of treatment by IL-1β and cotransfected C/EBP expression vector. Results are expressed as the means ± SEM of three independent experiments performed in duplicate.

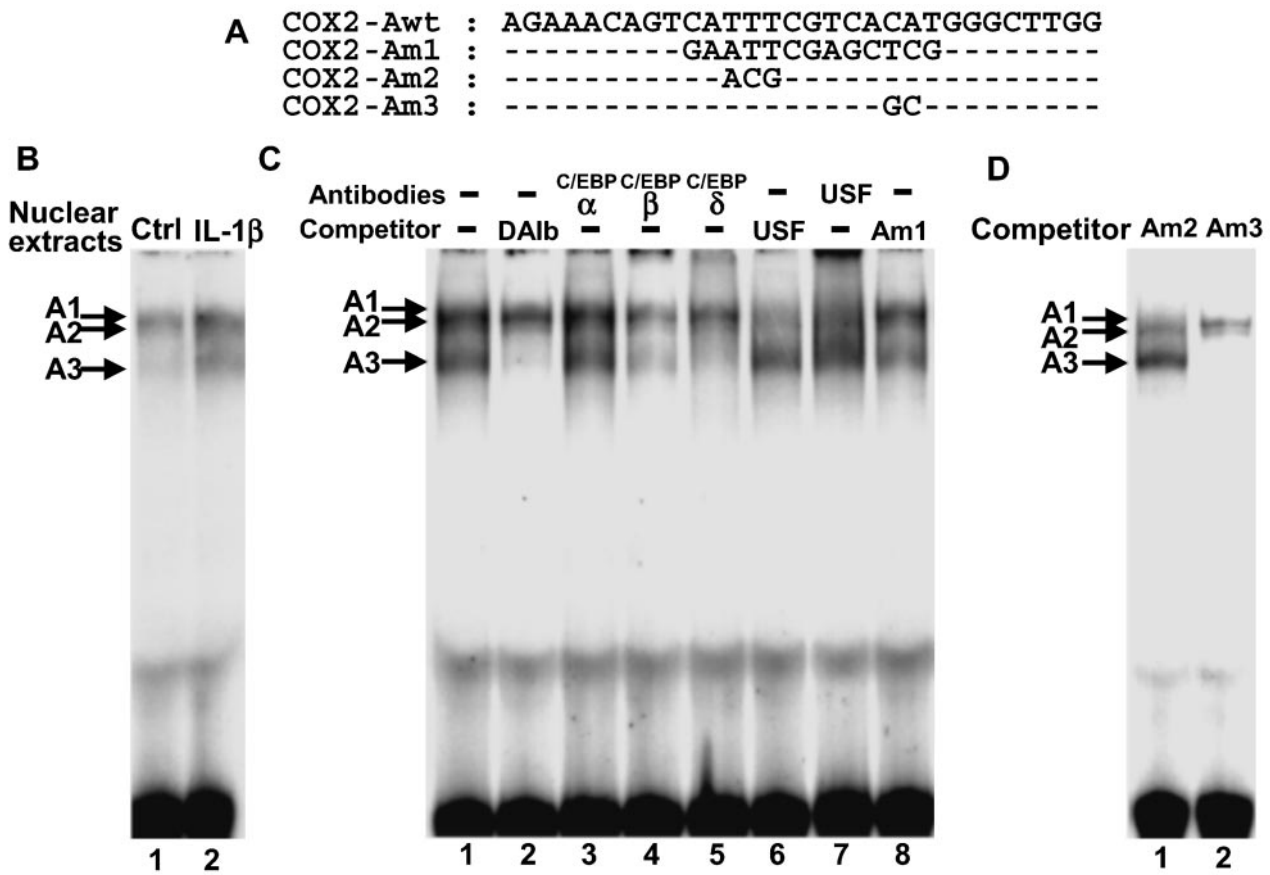


Fig. 3. The [−71; −42] COX2-A element binds C/EBPβ, C/EBPδ and USF factors. (A) Sequences of the wild type COX2-A oligonucleotide and its mutant COX2-Am1, COX2-Am2 and COX2-Am3. Dashes indicate the nucleotides unchanged in the mutant oligonucleotides. Nucleotides replacing the wild type sequence are indicated. (B) The COX2-A probe (100 000 cpm) was incubated with 6 μg of untreated-(lane 1) or IL-1β-treated chondrocytes nuclear extracts. (C) The COX2-A probe (100 000 cpm) was incubated with 6 μg of IL-1β-treated chondrocytes nuclear extracts. Unlabeled oligonucleotides D-Alb (lane 2), USF (lane 6) or COX2-Am1 (lane 8) were added to the nuclear proteins in 100-fold excesses over probe concentration and incubated for 15 min before adding the COX2-A probe. Specific antibodies to C/EBPα (lane 3) C/EBPβ (lane 4), C/EBPδ (lane 5) or USF (lane 7) were incubated with chondrocyte nuclear extracts for 15 min at 4 °C prior to adding poly(dI·dC). (D) As in (C) the COX2-A probe (100 000 cpm) was incubated with 6 μg of IL-1β-treated chondrocytes nuclear extracts. The unlabeled oligonucleotides COX2-Am2 (lane 1) or COX2-am3 (lane 2) were added to the nuclear proteins in 100-fold excesses over probe concentration and incubated for 15 min before adding the COX2-A probe. The complexes A1, A2 and A3 are indicated by arrows.

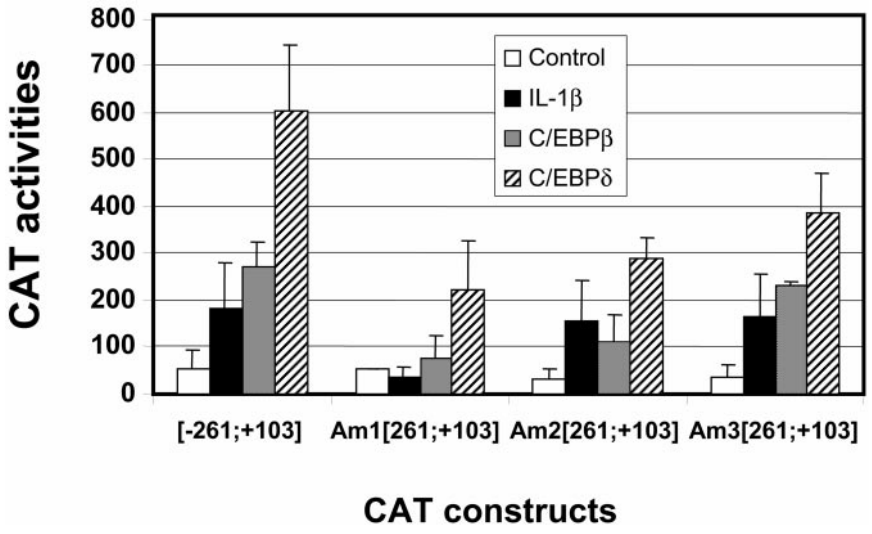


Fig. 4. Effects of mutations of the COX2-A element on stimulation of the activity of the human COX-2 promoter by IL-1β and C/EBP family members in rabbit chondrocytes. Primary cultures of rabbit chondrocytes were transfected as indicated in Fig. 1 with the wild-type [−261; +103]wt and mutant [−261; +103]Am1, [−261; +103]Am2 and [−261; +103]Am3 CAT constructs. Mutations in the COX2-A elements in the mutant CAT constructs are indicated in Materials and methods. Cells were grown with or without IL-1β (10 ng·mL^{-1}) as indicated in Fig. 1. The CAT construct were cotransfected with the PHD expression vectors containing C/EBPβ (dotted bars) or C/EBPδ (dashed bars) cDNAs. CAT activities were normalized by using β-galactosidase activities. Results are expressed as the means ± SEM of three independent experiments performed in duplicate.

stimulation by IL-1β, and its nuclear binding remains unchanged for up to 24 h, as shown by bandshift experiments using the enhancer of the light chain κ gene as a probe (KBEwt probe) (data not shown). The oligonucleotide corresponding to the [−233; −214] COX2-C element formed two complexes with nuclear extracts from IL-1β-treated chondrocytes, the major one having the higher electrophoretic mobility (Fig. 5A, lane 1). The lower mobility complex was only partially competed out by a 100-fold excess of unlabeled KBEwt oligonucleotide, while the high mobility one was totally displaced (Fig. 5A, lane 2). The higher mobility complex was supershifted by an antibody to p50 but not by an antibody to p65, indicating that it is formed by the p50/p50 homodimer (Fig. 5A, lanes 3 and 4). By contrast, the lower mobility complex was not afffected by incubating the chondrocyte nuclear extracts with the antibodies against p50 and p65 (Fig. 5A, lanes 3 and 4). These results show that the lower mobility complex is formed by a protein differing from the canonical p50 and p65 c-Rel family members. The oligonucleotide corresponding to the [−455; −433] COX2-D element also formed two complexes with the chondrocyte nuclear extracts (Fig. 5B, lane 1). Both these complexes were competed out by a 100-fold excess of unlabeled KBEwt oligonucleotide (Fig. 5B, lane 2). The upper complex must be generated by the p50/p65 heterodimer, because it was supershifted by the antibodies to both p50 and p65, while the lower one was supershifted only by the antibody to p50 (Fig. 5B, compare lanes 3 and 4), indicating that this complex was formed by the p50/p50 homodimer.

Various amounts of chondrocyte nuclear extract were incubated with the COX2-C, COX2-D and KBEwt probes to compare their affinity for NF-κB. The canonical KBEwt probe bound mainly to the p50/p65 heterodimer (Fig. 6, lanes 1–4). The complex formed between the KBEwt probe and the p50/p65 heterodimer was much more intense than either of the complexes formed between the COX2-D probe and 6–12 μg of nuclear extracts from IL-1β-treated chondrocytes (Fig. 6, compare lanes 2–4 to lanes 6–8). This result shows that the COX2-D element has a low affinity for NF-κB, especially for the transcriptionally active p50/p65 heterodimer. The COX2-C probe bound only the p50/p50 homodimer in chondrocytes nuclear extract concentrations of 6–12 μg (Fig. 6, lanes 9–12).

Mutation of the [−448; −449] 5′-GGGGATTCCC-3′ NF-κB site of the COX2-D element in the [−831; +103] fragment of the human COX-2 promoter did not modify the induction of

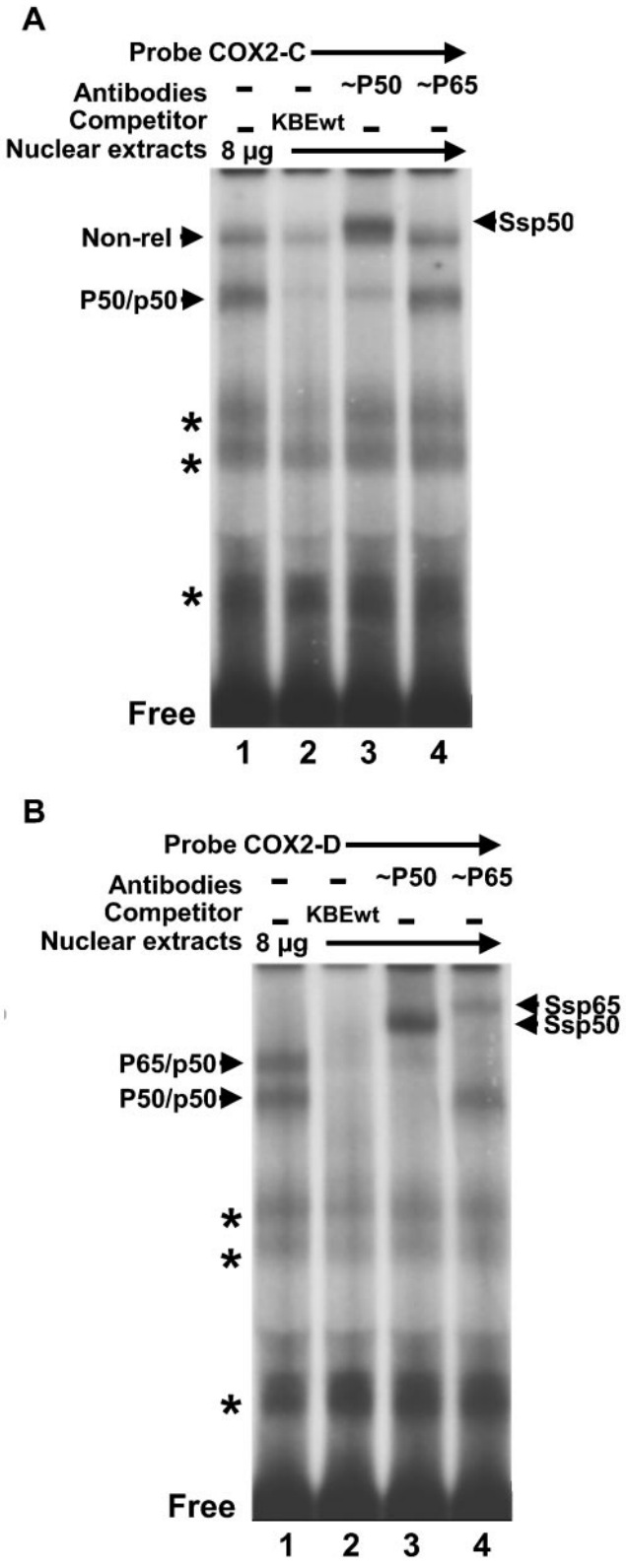


Fig. 5. The [−233; −204] COX2-C element binds the p50/p50 homodimeric forms of NF-κB and a nonrel factor while the [-455; −433] COX2-D element binds both the p65/p50 heterodimeric and p50/p50 homodimeric forms. (A) Bandshift assays were performed as described in Materials and methods. Primary cultures of rabbit chondrocytes were incubated with 10 ng·mL^{-1} IL-1β for 24 h prior to extraction of nuclear proteins. A 100-fold excess of unlabeled oligonucleotide KBEwt over the COX2-C probe concentration was added to the nuclear proteins for 15 min before adding the COX2-C probe (lane 2). Antibodies (1 μL of pure stock solution) against p50 (lane 3) or p65 (lane 4) were incubated with chondrocytes nuclear extracts for 15 min at 4 °C prior to adding poly(dI·dC). The complexes formed with the p50/p50 homodimeric form of NF-κB and the nonrel protein are indicated by the arrows. Ssp50 indicates the supershifted complexes in presence of the antibody to p50 (lane 3). Non-specific bands are indicated by asterisks. Electrophoresis was run on a 5% 30 : 1 bisacrylamide/acrylamide gel. (B) Bandshifts assays were performed as described in (A). A 100-fold excess of unlabeled oligonucleotide KBEwt over the COX2-D probe concentration was added to the nuclear proteins for 15 min before adding the COX2-D probe (lane 2). Antibodies (1 μL of pure stock solution) against p50 (lane 3) or p65 (lane 4) were incubated with chondrocyte nuclear extracts for 15 min at 4 °C prior to adding poly(dI·dC). The complexes formed with the p50/p65 heterodimer and the p50/p50 homodimer are indicated by arrows. Ssp50 and Ssp65 indicate the supershifted complexes in the presence of the antibodies to p50 and to p65, respectively. Non-specific bands are indicated by asterisks.

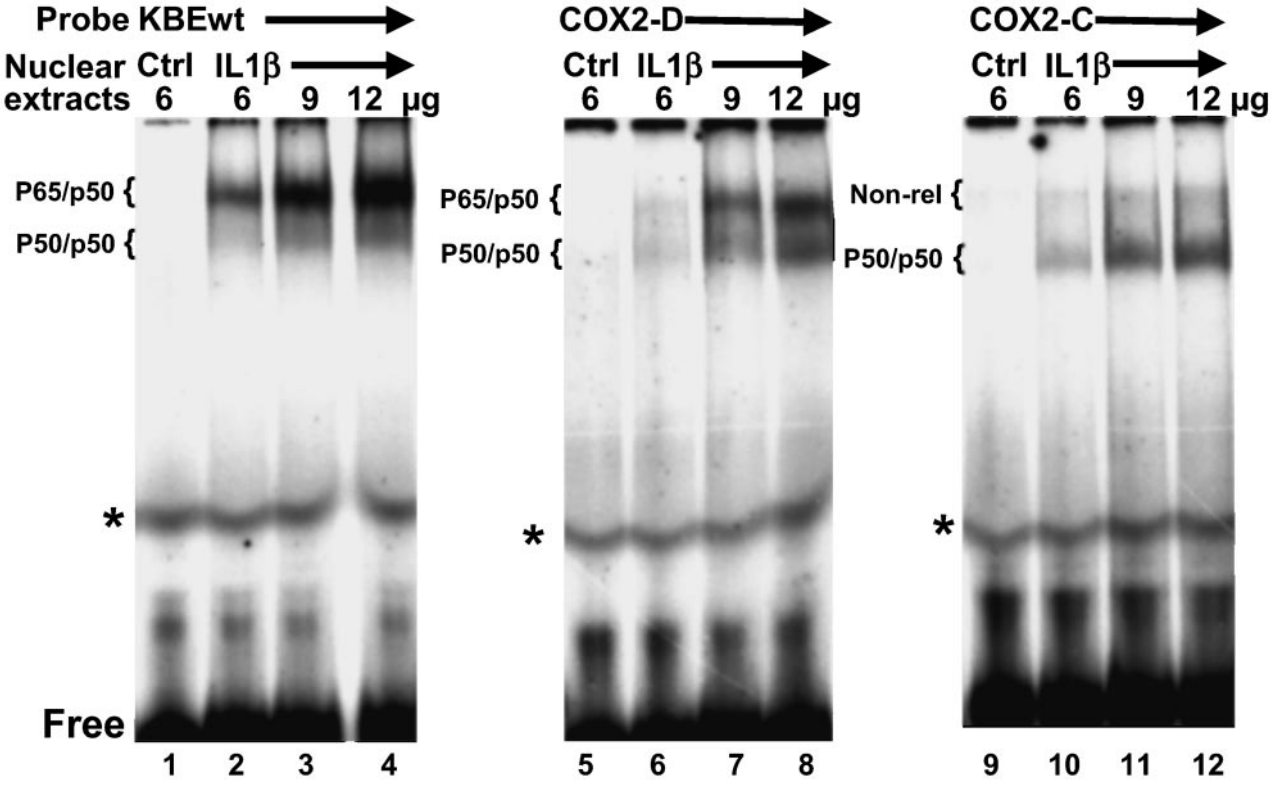


Fig. 6. The [−233; −204] COX2-C and [−455; −433] COX2-D elements have less affinity for NF-κB than the canonical KBEwt probe corresponding to the enhancer of the light chain κ gene. The 5″ end ^{32}P labeled KBEwt (lanes 1–4), COX2-D (lanes 5–8) and COX2-C (lanes 9–12) probes (100 000 cpm) were incubated with 3 μg poly(dI-dC) and 6 μg nuclear extracts of untreated chondrocytes (lanes 1, 5 and 9) or 6–12 μg (lanes 2–4, 6–8, 10–12) of IL-1β treated chondrocytes as indicated at the top of the figure. The complexes formed with the p50/p65 heterodimeric and p50/p50 homodimeric forms of NF-κB or with the nonrel protein are indicated by the arrows. Non-specific bands are indicated by the asterisk. Electrophoresis was run on a 6% 30 : 1 bisacrylamide/acrylamide gel.

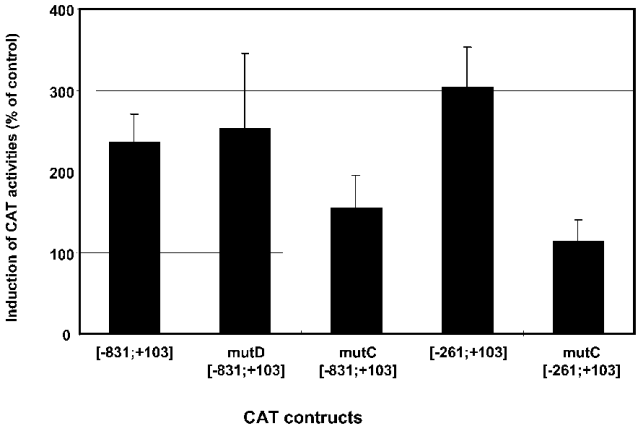


Fig. 7. Stimulation of the human COX-2 promoter by IL-1β in rabbit chondrocytes involves the p50/p50 homodimeric form of NF-κB bound to the COX2-C element. Rabbit chondrocytes were transfected as in Fig. 1 with the wild-type [−831; +103] wt, [−261; +103]wt CAT constructs or the mutant mutD [−831; +103], mutC [−831; +103] and mutC [−261; +103] CAT constructs. The NF-κB site in the COX2-D element was mutated in the mutD [−831; +103] CAT construct as indicated in Materials and methods. Similarly, the mutation of the COX2-C element in mutC [−831; +103] and mutC [−261; +103] CAT constructs are indicated in Materials and methods. Stimulations of the CAT activities by IL-1β were calculated relative to that induced by the corresponding CAT construct in the absence of treatment by IL-1β. Results are expressed as the means ± SEM of three independent experiments performed in duplicate.

transcription by IL-1β in primary cultures of rabbit chondrocytes (Fig. 7). By contrast, mutations of the [−223; −214] 5′-GGGACTACCC-3′ NF-κB site of the COX2-C element in the [−831; +103] and [−261; +103] fragments of the COX-2 promoter suppressed the stimulation of their activities by IL-1β in primary cultures of rabbit chondrocytes (Fig. 7). This indicates that, although it does not bind the transcriptionally p50/p65 active form of NF-κB, the COX2-C element plays a critical role in the regulation of the human COX-2 promoter by IL-1β in primary cultures of chondrocytes, in addition to the the C/EBP binding COX2-B element.

DISCUSSION

We have shown that C/EBPβ and C/EBPδ factors bind to the COX2-B element in chondrocyte primary cultures and that this binding is required for stimulation of the COX-2 promoter by IL-1β in chondrocytes, as both the 5′ deletion and the mutation of this sequence suppressed the effect of IL-1β. We have previously shown that IL-1β stimulates expression of the C/EBPδ gene without affecting that of the C/EBPβ gene [25]. Enhancements of the intensities of the complexes formed with the COX2-B probes in bandshift experiments are likely to be due to the increased C/EBPδ protein bound to DNA, either as a homodimer or a heterodimer with C/EBPβ. Such critical roles for C/EBPβ and C/EBPδ have been recently described by Wadleigh *et al*. [10] in the induction of the mouse COX-2 promoter activity by LPS in RAW 264.7 macrophages. These

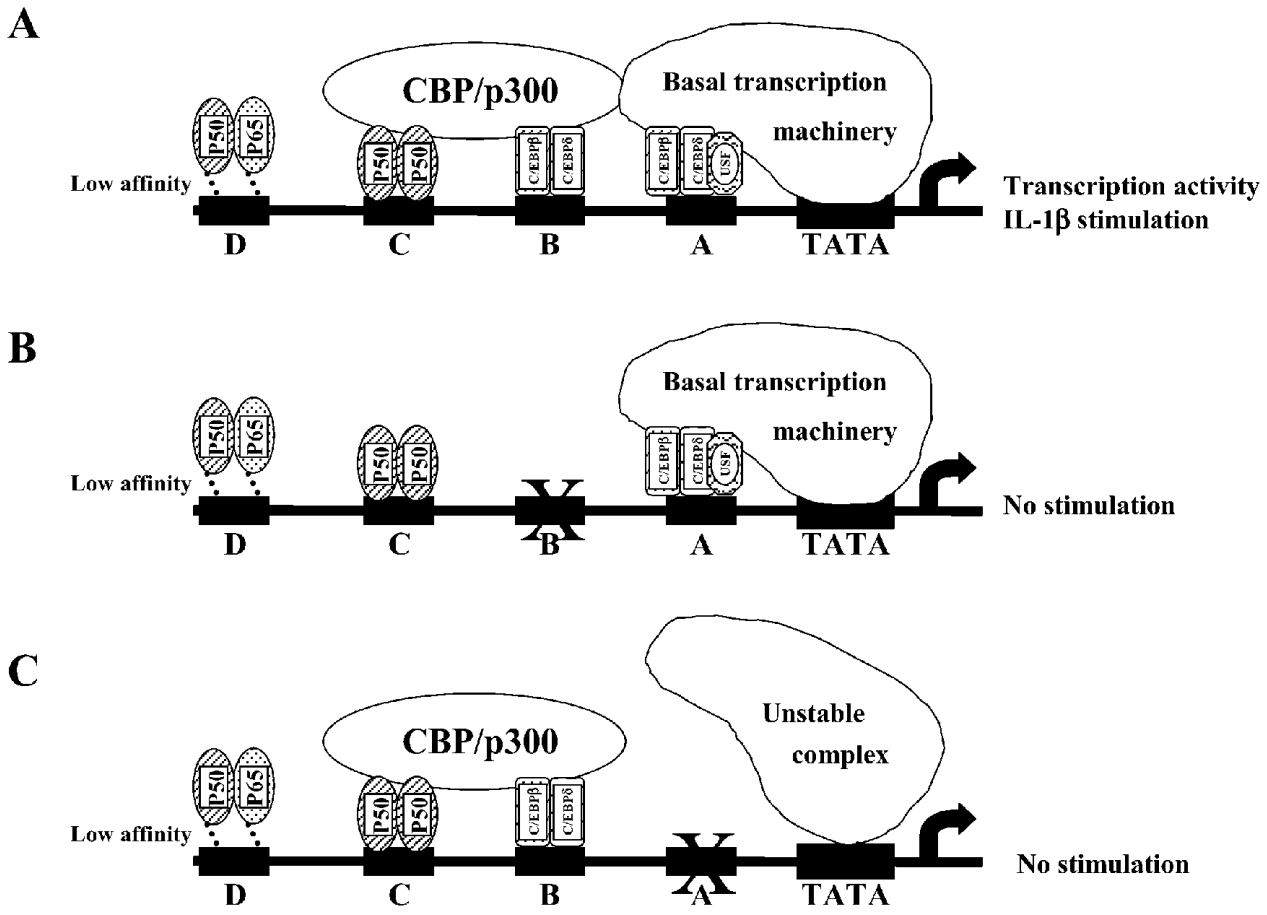


Fig. 8. Postulated interactions between the transcription factors bound to the regulatory elements of the human COX-2 promoter and the general transcription factors bound to the TATA box.

authors found two C/EBP binding sequences, one corresponding to the human distal site, the other located between the −138 and −130 positions [10]. The alteration of the first one reduced more drastically the induction of the promoter activity than the second one and mutations of both these sequences was required to abolish the endotoxin effect. These authors also demonstrated that a CRE sequence in a proximal element corresponding to the human COX2-A element is critical for the basal and stimulated activity of the mouse promoter while the adjacent E-box does not contribute to the transcription activity in RAW 264.7 macrophages [10].

We found no evidence of the presence of any C/EBP binding site in the human promoter between the COX2-B and COX2-A elements and deletion of the [−170; −81] fragment did not alter the activity of the human promoter in chondrocytes. By contrast, we demonstrated the binding of native C/EBPβ and C/EBPδ proteins to the COX2-A element in chondrocytes. Inoue *et al.* [13] also reported the binding of C/EBPδ to this element, but they used nuclei of vascular endothelial cells transfected by a C/EBPδ expression vector instead of nuclear extracts of native cells in their bandshift experiment. The binding of endogenous vascular endothelial nuclear proteins to the proximal regulatory element of the COX-2 promoter could have been prevented by the overproduced C/EBPδ in these experiments. In addition, bandshift experiments also demonstrated USF binding downstream of the C/EBP site in chondrocytes, although Morris and Richards [19] assumed that the COX2-A element of the human promoter would not bind USF family members because of a 1-bp mutation in the E-box compared with the homologous fragment of the rodent promoters. In contrast to the results obtained by Wadleigh *et al.* [10], the second C/EBP binding site, i.e. the COX2-A element, is not involved in the stimulation of the human promoter activity by IL-1β in chondrocytes. Surprisingly, exogenously expressed C/EBPβ and C/EBPδ factors stimulate the activity of mutant fragments of the COX-2 promoter lacking the COX2-B element but containing the COX2-A element. Furthermore, separated mutations of the C/EBP and USF binding sites in the COX2-A element did not alter the stimulation of the transcription activity by IL-1β or exogenously expressed C/EBP factors in chondrocytes while the mutation of both the sites, i.e. the Am1 mutation, drastically reduce these stimulations. We postulate that: (a) C/EBP factors bound to the COX2-B and COX2-A elements interact differently with the general transcriptional machinery; (b) the C/EBP and USF factors bound to the COX2-A element have complementary roles and may work together to transactivate the COX-2 promoter. USF factors regulate the transcription activity of the eukaryotic promoter through their interactions with components of the general transcription factors such as TFII-I [36] and TFII-D [37]. Interactions of C/EBPβ with TFII-B, another component of the transcription general machinery, has also been reported either as a direct interaction [38], or as an indirect interaction mediated by the nucleolar Nopp140 coactivator [39]. Multiple interactions between the numerous components of the general transcription machinery and the transcription factors bound to the proximal regions of

the promoter is the usual way of inducing high transcription activity. Alternative interactions of USF and C/EBP factors bound to the COX2-A element with different subunits of the general transcription machinery would explain why mutations of both the USF and C/EBP binding sites are required to decrease the basal promoter activity and to abolish its stimulation by IL-1β. In addition, exogenously expressed C/EBP factors could stimulate the activity of the promoter fragments containing the COX2-A element by increasing the number of interactions with components of the general transcription machinery. The location of the COX2-A element, 24 bp upstream of the TATA box, favors such direct interactions of C/EBP and USF factors with the general transcription machinery. By contrast, such interaction of the C/EBP proteins bound to the far upstream COX2-B element is unlikely and these C/EBP proteins might transactivate the COX-2 promoter in response to IL-1β through their interaction with the CBP/p300 coactivator [40]. IL-1β might not only increase the concentration of C/EBPδ as we previously demonstrated [25] but also qualitatively modify the interactions between C/EBP factors and CBP/p300. This could be mediated through post-translational modifications of CBP/p300 such as phosphorylations by MAP kinases, which have been previously evidenced [41]. Such hypothesis could explain why the COX2-B element is required for the stimulation of the human COX-2 promoter by IL-1β and the COX2-A element is not sufficient for this stimulation although it could be transactivated by exogenously expressed C/EBP factors. The possible regulatory mechanisms of the various factors that bind to the human COX-2 promoter are summarized in Fig. 8.

Many eukaryotic promoters, such as those of interleukin-8 [42], serum amyloid A [43], interleukin-6 [44], nitric oxide synthase [45], intercellular adhesion molecule-1 (ICAM) [46] or manganese superoxide dismutase [47] genes, are regulated through interactions between NF-κB and C/EBP factors. In most cases, these interactions involve p65. In chondrocytes, p50 but not p65 is implicated in the regulation of human COX-2 transcription by IL-1β. The present results show that the distal [−448; −439] sequence in the COX2-D element binds the p50/p65 heterodimer and the p50/p50 homodimer with a low affinity and that the proximal [−223; −214] sequence on the COX2-B element binds only the p50/p50 homodimer. Although it has not been previously reported in the cell types in which the regulation of the COX-2 promoter has been studied, this is consistent with the fact that the 3′ ends of the [−448; −439] 5′-GGGGATTCCC-3′ and [−223; −214] 5′-GGGACTACCC-3′ sequences do not fit with the perfect consensus binding site of NF-κB, such as the 5′-GGGACTTTCC-3′ sequence in the KBEwt oligonucleotide. The 5′ end of such a sequence is recognized by the p50 subunit, whereas the 3′ end is recognized by the p65 subunit or another p50 monomer [48]. Fujita *et al*. [35] reported that alteration of the 3′ end sequence decreases the affinity for the p50/p65 heterodimer much more drastically than its affinity for the p50/p50 homodimer. The formations of complexes with the homodimer p50/p50 are therefore favored *in vitro* when the 3′ end of the binding sequence is degenerated compared to the consensus site. Mutation of the COX2-C element suppressed the stimulation of the promoter activity by IL-1β although this element does not bind to the p50/p65 heterodimer but only to the p50/p50 homodimer. There are physical interactions between p50 and C/EBP factors [49–51] that would explain the requirement of both the COX2-B and COX2-C element for full stimulation of the human COX-2 promoter activity by IL-1β. The COX2-C elements also formed a faint complex with the chondrocyte nuclear extract, which was not altered by antibodies against p50 or p65. This complex was also observed by Crofford *et al*. [9] with nuclear extracts from IL-1β-treated human synoviocytes and by Schmedtje *et al*. [7] with nuclear extracts from hypoxemic human vascular endothelial cells but these authors did not further investigate the nature of the protein involved in its formation. MATINSPECTOR sofware and the TRANSFAC database [52] revealed homologies between the COX2-C element and consensus sites for several transcription factors, notably for the MZF1 factor, whose recognition site fits perfectly with the [−227; −220] 5′-AGTGGGGA-3′ sequence in this element. This factor, first described as lymphoid-specific [53], has been poorly studied. Additional studies will be required to determine the identity of the protein generating the low mobility complex with COX2-C element and its role in the regulation of the COX-2 promoter activity.

Increased synthesis of PGE_2 by chondrocytes and synoviocytes in response to IL-1β is a key event in the inflammatory process in joints. PGE_2 is produced through the interaction between COX-2 and $sPLA_2$ in cell types that express both these genes in response to stimulation by IL-1β [54,55]. As a consequence, blocking the expression of both these genes would reduce PGE_2 production, inflammation and the resulting degradation of cartilage. We have shown [25] that C/EBPβ and C/EBPδ are responsible for inducing $sPLA_2$-IIA transcription in primary culture chondrocytes. The present paper demonstrates that these factors coregulate the COX-2 transcription in these cells. These transcription factors are therefore potential targets for anti-inflammatory therapy in joints.

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