Induction of Secreted Type IIA Phospholipase A2 Gene Transcription by Interleukin-1β

ROLE OF C/EBP FACTORS*

Received for publication, February 15, 2000, and in revised form, April 26, 2000
Published, JBC Papers in Press, May 1, 2000, DOI 10.1074/jbc.M001250200

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Secreted type IIA phospholipase A_2, which is involved in arachidonic acid release, is abundantly produced by chondrocytes and secreted in the synovial fluids of patients affected by rheumatoid arthritis. Transfection experiments showed that interleukin-1β stimulates the phospholipase A_2 (−1614; +20) promoter activity by 6-7 fold and that the [−210; −176] fragment is critical for this stimulation. CAAT enhancer-binding protein (C/EBP) β and C/EBPβ transcription factors bind to this element as shown by bandshift experiments. Interleukin-1β increased the levels of C/EBPβ mRNA as soon as 2 h and up to 24 h without affecting those of C/EBPβ. Higher amounts of C/EBPβ proteins correlate with the stimulation of C/EBPβ mRNA. Mutations or 5 deletions in the upstream [−247; −210] region reduced by 2-fold the basal and interleukin-1β-stimulated transcription activities. Two types of factors bind to overlapping sequences on this fragment: NF1-like proteins and the glucocorticoid receptor. The glucocorticoid receptor is responsible for a moderate stimulation of the promoter activity by dexamethasone and may interact with C/EBP factors to achieve a full transcription activity in basal conditions and in the presence of interleukin-1β. A [−114; −85] proximal regulatory element forms three complexes in bandshift experiments, the slowest mobility one involving the Sp1 zinc finger factor. Mutation of this sequence reduced to 2-fold the stimulation of the promoter activity by interleukin-1β or the C/EBP factors. Induction of the transcription of secreted type IIA phospholipase A_2 gene by interleukin-1β in chondrocytes absolutely requires C/EBPβ and C/EBPβ factors but does not involve NF-κB.

The synovial fluid of patients suffering from rheumatoid arthritis or osteoarthritis contains large quantities of prostaglandin E2. Such quantities inhibit collagen synthesis and therefore contribute to joint destruction. These inflammatory lipid mediators are produced by a cascade of enzymes among which phospholipases A_2 (PLA_2) play a key role by releasing arachidonic acid from membrane phospholipids. Two calcium-dependent PLA_2 are involved in the release of arachidonic acid. Cytosolic PLA_2, a ubiquitous 85-kDa enzyme, is activated by MAP kinases and translocated from the cytosol to membrane (1, 2). Type IIA secreted PLA_2 (sPLA_2-IIA) was originally purified from the synovial fluid of patients with rheumatoid arthritis, which contains high quantities of this enzyme (3). sPLA_2-IIA belongs to a large group of 13–15-kDa secreted enzymes present in mammalian fluids and in the venoms of snakes and insects. According to a recent classification (4), the pancreatic version of the enzyme has been included in a type I group whereas the synovial PLA_2 is referred as type IIa sPLA_2. Three other recently cloned mammalian PLA_2 were classified in IIC, V, and X groups, but their involvement in the arachidonic acid release remains unknown.

Purified or recombinant sPLA_2-IIA triggers joint inflammation when it is injected intra-articularly in rabbits (5). The number of rheumatoid arthritis-affected joints and the presence of destructive erosion have been correlated with the amount of sPLA_2-type IIA in the serum of patients (6). Mice with both the TNFα and sPLA_2-II transgenes exhibit more joint destruction than those with TNFα alone (7). Interleukin-1β (IL-1β) is the most abundant cytokine in inflammatory synovial fluids. It stimulates the expression of numerous genes in articular cells (8) and increases the level of sPLA_2-IIA mRNA in chondrocytes. We have previously demonstrated that prostaglandin E2 production by rabbit articular chondrocytes is related to plasma membrane-associated sPLA_2-IIA activity and that the transcription rate of sPLA_2-IIA gene is stimulated by IL-1β (9–11).

Three main classes of transcriptional factors have been shown to mediate the effect of IL-1β on gene transcription: (i) NF-κB is a dimer of p50 and p65 subunits and belongs to the Rel family. (ii) A member of the STAT family of transcription factors, which are activated through phosphorylation by Jak kinases and translocated to the cell nucleus within a few minutes, was characterized as an IL-1β-stimulated factor but can also be activated by IL-6 and lipopolysaccharide (12). (iii) AP-1 can also be involved in the IL-1β pathway because IL-1β induces the transcription of the c-Jun and c-Fos genes in some cell models (13).

C/EBP transcription factors are involved in the regulation of gene transcription by IL-6. The C/EBP family includes three main members: C/EBPa, C/EBPβ, and C/EBPδ. This last member is transcriptionally induced by IL-6 (14, 15), whereas C/EBPβ is mainly regulated at the post-transcriptional level by this cytokine in hepatoma cell lines (16, 17), although a trans-
scriptional regulation of C/EBPβ by the inflammatory cytokines has also been described (for review see Ref. 18). In contrast to IL-6, the relationship between IL-1β and C/EBP factors has been poorly studied, and some positive regulations in interaction with NF-κB have been reported (19, 20). However, induction of C/EBP binding to DNA by pro-inflammatory cytokines correlates with the accumulation of prostaglandin E2, and both effects are reversed by anti-inflammatory cytokines (21). C/EBP factors act with NF-κB to induce the transcription of many acute phase response genes in response to pro-inflammatory cytokines, and this effect is based on direct protein-protein interactions (22, 23). Similar interactions have also been reported between C/EBP proteins and the glucoorticoid receptor (GR), which may explain the co-induction of the transcription of acute phase response genes by glucoorticoids and cytokines (24). The GR is a member of the steroid/nuclear receptor superfamily and binds to the glucocorticoid-responsive element (GRE) on gene promoters (25).

We have previously shown that the activity of the sPLA₂-IIA promoter is controlled by three regulatory elements in human hepatoma HepG2 cells (26) (see Fig. 1A). The [−210; −176] element C is critical for the stimulation of the promoter by IL-6 and binds C/EBP family members, whereas an adjacent [−247; −210] element D is recognized by several factors, some of which belong to the NF1 family (27). The [−114; −85] element B is responsible for a high basal activity when the region upstream of the C/EBP-binding site is deleted (28). We have demonstrated that C/EBP factors can mediate the stimulation of transcription by IL-6 in HepG2 cells by suppressing the basal inhibition of transcription, a process that may involve single stranded binding activities (27).

In this study, we have identified the sequences and transcription factors involved in the stimulation of the sPLA₂-IIA promoter by IL-1β in chondrocytes. We have found that C/EBPβ plays a critical role in this stimulation and is transcriptionally induced by IL-1β (26). 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. (26).

**Bandshift Assays**—We used the double-stranded CWT and DWT as wild type probes, corresponding to the [−210; −176] sequence of the element C and to the [−247; −210] sequence of the element D. These double-stranded oligonucleotides (100 ng) were labeled with radioactive phosphate and 50 μCi of [γ-32P]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 centrifuge (Beckman). The supernatants were collected, and the protein concentrations were measured according Olivier et al. (26).

**Plasmid Constructions and Chondrocyte Cultures and Transfections**—The various CAT constructs containing wild type and mutant fragments of the sPLA₂-IIA promoter have been described elsewhere (26, 27). PHD expression vectors containing C/EBPβ and C/EBPβ were a gift from Dr. Ciliberto (Rome, Italy).

Three-week-old female Fauve de Bourgogne rabbits were killed and the shoulder blades and femoral heads were dissected out under sterile conditions as described by Jacques et al. (9). The articular cartilage was removed, cut into small pieces, and digested at 37 °C with 0.05% hyaluronidase in Gey medium for 15 min and then with 0.25% trypsin for 30 min and finally with 0.2% collagenase for 90 min. The chondrocytes were then washed with Ham's F-12 medium for 60 min. The suspension of chondrocytes was seeded into 60 mm dishes (1.5 × 10^5 cells per dish) in Ham's F-12 medium supplemented with 10% fetal calf serum. The cells were maintained at 37 °C in 5% CO₂, and the culture medium was changed every 2–3 sday. The cells reached preconfluency within 6–7 days.

Chondrocytes were transfected using the calcium phosphate DNA co-precipitation method. Cells were changed by Dulbecco's modified Eagle's medium before transfection. Cells were incubated with the transfection mixture containing 12 μg of pUC-SH-CAT constructs and 2.5 μg of plasmids bearing the β-galactosidase gene for 4 h and then shocked with HBS buffer (21 ml HEPES, pH 7.1, 16 mM dextrose, 0.8 mM Na₂HPO₄, 5 mM KCl, and 157 mM NaCl) containing 15% glycerol for 20 min. The cells were incubated at 37 °C for 20 h in Ham's F-12 supplemented with 0.2% bovine serum albumin and grown for an additional 24 h in the presence or absence of IL-1β (10 ng/ml). The harvested cells were lysed by incubation with 50 μl of 100 mM Tris, pH 7.8, 0.7% Nonidet P-40 for 15 min at 4 °C. CAT activities were measured by the two-liquid phases method as described by Fan et al. (27). 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.

**Preparation of Nuclear Extracts and Cell Lysates**—Chondrocytes nuclear extracts were prepared as described previously (27). Briefly, confluent cells from 3 T100 dishes were harvested in Ham's F-12 without fetal calf serum and then incubated in the presence or absence of IL-1β (10 ng/ml) for 24 h in Ham's F-12 containing 10% fetal calf serum. They were washed and scraped into phosphate-buffered saline. The cells were centrifuged at 1500 × g for 5 min, and the pellet was suspended in 500 μl of buffer A (5 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 50 mM NaF). The cells were incubated at 4 °C for 15 min, centrifuged at 6000 × g for 10 min, and the pellet was suspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 2.5 mM KCl, 0.5 mM NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml 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). The supernatants were collected, and the protein concentrations were measured according Olivier et al. (26). 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. (26).

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measured by spectrophotometry, and its integrity was assessed by agarose gel electrophoresis; 15 μg of total RNA/lane were separated on 1% agarose/2.2% formaldehyde gels and transferred to nylon filters (Hybond N; Amersham Pharmacia Biotech). The membranes were prehybridized for 15 min, then hybridized at 65°C for 2 h with the various probes in the rapid hyb-buffer medium (Amersham Pharmacia Biotech). The specific C/EBPβ and C/EBPδ probes were obtained by digestion of the corresponding expression vectors with PstI and XhoI (New England Biolabs, Boston, MA) respectively. The digestion products were separated on 1% agarose gels, the 500- and 850-bp-length bands corresponding to fragments of the C/EBPβ and C/EBPδ cDNA, respectively, were sliced and extracted using the Gene-Clean kit (New England Biolabs, Boston, MA). The abundance of C/EBPβ protein in nuclear extracts from rabbit primary culture chondrocytes (Bio101, La Jolla, CA). The C/EBP probes were labeled using the random-prime labeling system (Amersham Pharmacia Biotech) and [α-32P]dCTP (3000 Ci/mmol). An oligonucleotide hybridizing to the 28 S RNA was labeled with [γ-32P]ATP by T4 kinase and used as probe to take into account the variations in loaded and transferred RNA. The hybridized filters were washed twice in 2× SSC (150 mM NaCl, 17 mM trisodium citrate) 0.1% SDS at room temperature for 15 min and then twice in 0.1× SSC, 0.1% SDS for 15 min at room temperature and at 50°C. Autoradiography was performed for 10 days for C/EBPβ probes and 24 h for the 28 S RNA probe. The blots were successively hybridized with the C/EBPδ, C/EBPβ, and 28 S RNA probes. The filters were washed in 0.1× SSC, 0.1% SDS at 85°C for 10 min before rehybridization.

Western Blotting—Aliquots (50 μg) of nuclear proteins were separated on a 12% SDS-polyacrylamide gel electrophoresis in 0.38 M Tris-HCl, 0.1% SDS, pH 8.5, and electrophoretically transferred to Protran BA83 nitrocellulose membranes (Schleicher & Schull). The membranes were saturated in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 5% nonfat milk at 4°C overnight, hybridized with anti-C/EBPβ or anti-C/EBPδ antibodies (Santa Cruz) for 1 h at 4°C, washed in the saturation buffer, and then developed using the ECL system (Amersham Pharmacia Biotech). The abundance of C/EBP protein in nuclear extracts from untreated and IL-1β-treated chondrocytes was calculated by quantitative scanning of autoradiograms using a CCD video camera and the Denslab system (Quantum Bioprobe, Montreuil, France).

RESULTS

The Regulatory Element C of the sPLA2-IIA Promoter Is Critical for the Stimulation of Its Activity by IL-1β and Binding C/EBPβ and C/EBPδ—Rabbit primary culture chondrocytes were transfected with CAT constructs containing various 5′ deleted fragments of the sPLA2-IIA promoter. A 24-h treatment of the cells by IL-1β increased by 6.6 ± 2 the activity of the [−1614; +20] fragment of the sPLA2-IIA promoter (Fig. 1B). Deletion of the [−247; −225] fragment decreased by 2-fold the basal and IL-1β-stimulated transcription activities. The relative stimulation of the transcription activities by IL-1β was not significantly modified by the 5′ deletions from the −225 to the −203 positions because the basal and stimulated activities were similarly decreased. Deletion downstream from the position −195 completely suppressed the stimulation of the transcription activity by IL-1β. Deletion of the −195 to −135 fragment produced an additional 2-fold reduction of the transcription activity, and the promoter activity was suppressed by a further deletion to the −87 position (Fig. 1B).

Electrophoretic mobility shift assays showed that chondrocyte nuclear extracts formed two complexes, C1 and C2, with the CWT probe, which corresponds to the [−210; −176] fragment (Fig. 2B, lanes 1 and 7). The slowest electrophoretic mobility complex C1 could be clearly observed only when the chondrocytes were treated with IL-1β prior the extraction of the nuclear proteins (Fig. 2B, compare lanes 1 and 7). The C2 complex was formed when the extracts of both the untreated and IL-1β-stimulated chondrocytes were used, but its intensity was higher when the cells were treated by IL-1β (Fig. 2B, compare lanes 1 and 7). Because this region was previously shown to correspond to a C/EBP-binding site in hepatocytes (26), we used as competitors the C/EBP-binding oligonucleotide corresponding to the D element of the murine albumin promoter (32) and the Cmut oligonucleotide in which the −199/−197 5′-TTT-3′ triplet was mutated into an 5′-GCC-3′ sequence (Fig. 2A). All the complexes were suppressed when the chondrocyte nuclear extracts were preincubated with the C/EBP-binding oligonucleotide prior to the addition of the CWT probe (Fig. 2B, lanes 2 and 8). By contrast, none of the complexes were com-
peted out by the Cmut oligonucleotide (Fig. 2B, lanes 3 and 9). An antibody raised against C/EBPα did not alter any of the complexes formed between the CWT probe and the chondrocyte nuclear extracts (Fig. 2B, lanes 4 and 10). Because all the C/EBP family members share the same binding site, it is likely that rabbit articular chondrocytes lack C/EBPα expression. Antibodies to C/EBPβ and C/EBPδ supershifted the C1 complex (Fig. 2B, lanes 7, 81, 11, and 12). The upper part of the C2 complex was displaced by the antibody to C/EBPβ complex (Fig. 2B, lanes 5 and 11), whereas its lower part was shifted by the antibody to C/EBPδ (Fig. 2B, lanes 6 and 12). Because C/EBPβ and C/EBPδ bind to DNA by forming heterodimers as well as homodimers, these data indicate that the C1 complex is formed by C/EBPβ-C/EBPδ heterodimers, whereas the C2 complex is heterogeneous and corresponds to co-migrating C/EBPβ-C/EBPβ and C/EBPβ-C/EBPδ homodimers.

The mutations of the whole −204; −181 sequence or the −199/−197 triplet abolished the stimulation of the promoter activity by IL-1β (Fig. 3). The 6-fold stimulation of the wild type −326; +20 sPLA₂-IIA promoter activity by IL-1β was mimicked by co-transfecting the C/EBPβ and C/EBPδ expression vectors with the −326; +20-pUC-SH-CAT construct in the absence of cytokine treatment (Fig. 3). By contrast co-transfection had no effect with the mutated C/EBPβ and C/EBPδ factors (Fig. 3), suggesting a pivotal role for the C/EBP factors in the induction of the sPLA₂-IIA promoter activity by IL-1β.

IL-1β Stimulates the Expression of the C/EBPβ Gene—Messenger RNA levels of C/EBPβ and C/EBPδ were measured in chondrocytes at various times after treatment by IL-1β. The C/EBPβ mRNAs were barely detectable by Northern blot in absence of IL-1β. The C/EBPδ mRNA levels were increased by IL-1β after a 2-h treatment, reached maximal values between 14–16 h of treatment, and were maintained over a 24-h period. By contrast IL-1β did not affect the levels of C/EBPδ mRNAs (Fig. 4). Western blot experiments confirmed that IL-1β increased by 3–4-fold the concentration of C/EBPδ proteins (Fig. 5).

The −247; −210 Regulatory Element D Binds CTF/NF1 Family Members and the Glucocorticoid Receptor, Which Potentiates the Transactivation of the sPLA₂-IIA Promoter by the C/EBPβ and C/EBPδ Factors—The −247; −210 element D displayed a consensus CTF/NF1 hemi-site 5'-TGGCA-3' located between the positions −224 and −220 and overlapping an upstream −229; −224 5'-TGTCTT-3' sequence, which is homologous to the consensus 5' half-site for the glucocorticoid receptor 5'-TGTTCT-3' (35) (Fig. 6A). The DWT probe (Fig. 6A) formed, with nuclear extracts from untreated chondrocytes, two specific complexes, D1 and D2 (Fig. 6B, lanes 1 and 2). The formation of complex D1 was suppressed by the addition of a 250-fold excess of a consensus GRE (Fig. 6B, lane 4), which suggests that this complex correspond to the binding of the glucocorticoid receptor. Complex D2 was competed out by the same excess of a NF1-binding oligonucleotide, which indicates the involvement of CTF/NF1 family members in the formation of this complex (Fig. 6B, lane 3).

To further delineate the binding sites of the NF1 proteins and the glucocorticoid receptor, four oligonucleotides corresponding to a mutation of the element D (Fig. 6A) were used in bandshift experiments. The Dm1 oligonucleotide, in which the −224; −220 CTF/NF1 hemi-site was mutated (Fig. 6A), competed out the D1 complex but did not affect the formation of the D2 one (Fig. 6B, lane 5), confirming that this latter involves CTF/NF1 family members. The Dm2 oligonucleotide which contains two mutations, respectively, on the −228 G nucleotide and on the −238/−236 sequence (Fig. 6A), competed out the D1 complex but not the D2 one (Fig. 6B, lane 6). The involvement of the −229; −224 5'-TGTCTT-3' sequence in the formation of the D1 complex was confirmed by the lack of competition of this
complex by the Dm3 oligonucleotide (Fig. 6B, lane 7) in which the −229/−228 TG nucleotides were mutated into CA (Fig. 6Aa). In addition the 250-fold excess of Dm3 oligonucleotide did not fully displaced the D2 complex, indicating that the sequence upstream from the −224; −220 CTF/NF1 hemic-site plays a role in the binding of the CTF/NF1 family members to the element D (Fig. 6B, lane 7). Finally the mutation of the whole −231; −224 5′-TGTGTTTTT-3′ sequence abolished the ability of the Dm4 oligonucleotide to compete with the DWT probe for the formation of both the D1 and D2 complexes (Fig. 6B, lane 8). The Dm1 oligonucleotide was also used as probe and formed the D1 complex but not the D2 one (Fig. 6C, lane 3). The GRE probe formed two complexes with the nuclear extracts from untreated and IL-1β-stimulated chondrocytes. The upper complex, which was the most intense, co-migrated with the D1 complex formed with the DM1 probe (Fig. 6C, compare lanes 1 and 2 with lane 3). This result supports the involvement of the glucocorticoid receptor in the formation of the D1 complex and also indicates that this receptor is present in nuclei in absence of dexamethasone treatment. The Dm3 oligonucleotide used as probe did not form either the D1 or the D2 complex, confirming the involvement of the sequence upstream the −224; −220 CTF/NF1 hemic-site in the binding of the CTF/NF1 family members to the D element (Fig. 6C, lane 4). Treatment of chondrocytes with IL-1β for 24 h did not affect the formation or the intensity of the complex formed with the GRE probe (Fig. 6C, lane 2).

A 24-h treatment of transfected chondrocytes by dexamethasone (10−7 M) induced a moderate stimulation of the −326; +20 sPLA2-IIA promoter activity by 160 ± 12%, and this stimulation was suppressed by co-incubation with the synthetic glucocorticoid analog and inhibitor RU486 (10−5 M) (Fig. 7A). Co-incubation with dexamethasone (10−7 M) had no effect on the stimulation of the −326; +20 sPLA2-IIA promoter activity by IL-1β (Fig. 7A). Similarly, RU486 did not modify the induction of transcription activity by IL-1β (Fig. 7A). Dexamethasone elicited a 5-fold induction of a promoter containing a GRE sequence upstream of the thymidine kinase promoter, and this induction was inhibited by the anti-glucocorticoid RU486. The dexamethasone-stimulated transfection was equivalent in the case of the GRE-TK and −326/+20 CAT construct, whereas the basal activities of these two plasmids were different.

Stimulation of the sPLA2-IIA transcription by dexamethasone in transient transfection experiments of chondrocytes was suppressed by the deletion of the region upstream the −225 position (Fig. 7B), which altered the GRE (Fig. 6A). This last 5′ deletion also decreased the basal transcription activity as previously shown in Fig. 1B. The substitution of the −231; −224 5′-TGTGTTTTT-3′ fragment overlapping the NF1 and GR hemisites by the nonspecific sequence 5′-GTTACCCG-3′ drastically reduced the basal transcription activity of the resulting promoter and suppressed its stimulation by IL-1β and dexamethasone (Fig. 7B). The mutation of the 5′-TG-GCA-3′ CTF/NF1 hemic-site in the −223; −218-pUC-SH-CAT construct did not affect either the basal transcription activity or its stimulation by IL-1β but abolished that by dexamethasone (Fig. 7B). Taken together, these results indicate that the stimulation of the sPLA2-IIA transcription by IL-1β and dexamethasone are not mediated in the same way, although both involve the GR hemic-site on the element D. The GRE located on the element D is required to achieve full basal and IL-1β-stimulated transcription activities.

The Zinc Finger Protein Sp1 Is Involved in the Chondrocyte Nuclear Proteins Bound to the (−114; −87) Regulatory Element B of the sPLA2-IIA Promoter—We have demonstrated that the structures [−107; −99] 5′-GACCACGCC-3′ sequence is critical for the sPLA2-IIA promoter activity in HepG2 cells (28). The BWT probe, which corresponds to the −114; −87 sequence of the sPLA2-IIA promoter (Fig. 8A), formed three complexes with the chondrocyte nuclear proteins (Fig. 8B, lane 1). A 500-fold excess of the unlabeled BMut oligonucleotide, in which the −107; −99 sequence was mutated (Fig. 8A), did not displace any of the complexes B1, B1′, or B2 (Fig. 8B, lane 3). The complex B1 was supershifted by a specific antibody to Sp1 (Fig. 8B, lane 5), indicating the involvement of Sp1 in the formation of this complex. The pretreatment of the chondrocytes by IL-1β did not change the mobility or the intensity of any of the three complexes formed with the BWT probe (Fig. 8B, lane 6).

Transfection experiments showed that the mutation of the native [−107; −99] sequence in the −114; −87-pUC-SH-CAT plasmid reduced the stimulation of the −326; +20 promoter activity by IL-1β 2-fold (Fig. 9). This mutation had a moderate effect on the basal activity of the sPLA2-IIA promoter.
activity, i.e. a decrease by 30% was observed (Fig. 9). When the expression vectors of C/EBPδ was co-transfected with the constructs containing the wild type and the mutant promoters, respectively, the stimulation of the transcription activity dropped from 7–8-fold to 2.5–3.5-fold. By contrast, overexpressed C/EBPδ stimulated the activity of the wild type promoter 7–8-fold and that of the mutant promoter 5-fold (Fig. 9). These results show that the factors bound to the regulatory element B are involved in the stimulation of the sPLA2-IIA promoter activity by IL-1β and reinforce the transactivation by the C/EBP factors.

**DISCUSSION**

We present evidence that C/EBP factors are central to the control of sPLA2-IIA gene transcription by IL-1β. The major complex C2, formed by C/EBPδ and C/EBPδ homodimers, was observed with extracts from both untreated and IL-1β-treated chondrocytes. The amounts of C/EBPδ mRNA and protein in rabbit chondrocytes are increased by IL-1β, which does not, in contrast, modify the amounts of C/EBPβ mRNA. The newly synthesized C/EBPδ protein can form higher amounts of homodimers and heterodimers with C/EBPβ, and this could explain the fact that the intensities of both the C1 and C2 complexes were reinforced when the nuclear proteins were extracted from IL-1β-treated chondrocytes. The stimulation of sPLA2-IIA transcription by IL-1β is therefore mediated through the increase of the C/EBPδ gene expression itself. The increase of C/EBPδ mRNA levels after 2 h is
consistent with the delayed increase of the sPLA2 mRNA levels, which we previously measured in rabbit primary culture chondrocytes (9).

Although the element C is the key element for the regulation

of sPLA2-IIA promoter activity by IL-1β in chondrocytes, intact regulatory elements B and D are required to achieve full basal and stimulated transcription activity. We found that the mutation of the [-107; -99] sequence abolished the binding of nuclear proteins to the element B and strongly reduced the stimulation of the activity of sPLA2-IIA promoter by IL-1β. However, overexpression of C/EBPβ compensates for the effect
of the mutation of the \([-107; -99]\) sequence. This suggests that C/EBP\(\beta\) and C/EBP\(\delta\) may interact differently with the general transcription machinery in terms of their affinity for the various co-activators such as CBP/P300 (34) or Nopp140 (35). We have shown that the 5'-TGTGTTT-3' sequence of element D is critical for the formation of a slow electrophoretic mobility complex D1 and for full sPLA\(_2\)-IIA basal and dexamethasone- and IL-1\(\beta\)-stimulated transcription activity. The D1 complex is competed out by a consensus GRE and co-migrates with the main complex formed between the GRE probe and the chondrocyte nuclear extracts. The glucocorticoid receptor typically binds to two hexameric inverted repeats separated by 3 bp. The \([-229; -224]\) 5'-TGTGTTT-3' sequence of element D is highly homologous to the consensus 3' hemi-site 5'-TGTGTT-3' for GREs. The G and T nucleotides on positions 2 and 3, which interact with Arg-447 and Val-443 residues of GR, are conserved, and we have observed in a previous study that the -228 G nucleotide is a major site of interference with a liver protein forming an intense complex with a mobility similar to that of the D1 complex (26). However, the C nucleotide on position 5, which interacts with the Lys-442 residue of GR in consensus GRE (36) was replaced by a T. The element D lacks a typical 5' half-site, but the \([-241; -236]\) 5'-CTGCCCT-3' sequence, which is 6 bp upstream of the 5'-TGTGTTT-3' site, is weakly homologous to the consensus sequence (36). Furthermore, variations of orientation and spacing between the two hemi-sites up to 8–9 bp have been described (37, 38), and many GRE lack 5' hemi-sites, such as those of mouse IL-2 receptor \(\alpha\), human elastin, and rat chromogranin promoters (39–41).

We have shown that both the GRE and DWT probes form complexes with nuclear extracts from untreated chondrocytes. This indicates that the glucocorticoid receptor was present in chondrocyte nuclei in the absence of treatment by dexamethasone or IL-1\(\beta\). Several studies have reported the localization of unliganded glucocorticoid receptors inside the nuclei of several cell types (42–45). More recently, two types of glucocorticoid receptor have been described in humans, hGR\(\alpha\) and hGR\(\beta\), which differ at their C-terminal end beyond amino acid 727; hGR\(\alpha\) has 50 additional residues; and hGR\(\beta\), which contains 15 nonhomologous residues that are generated through alternative splicing of the last exon (46). hGR\(\alpha\) is sequestered in the cytosol by heat shock protein hsp90 in basal conditions, binds glucocorticoids or RU486, and is translocated into the nuclei; hGR\(\beta\) in the nuclei does not bind dexamethasone or RU486 but can bind to GRE (46, 47). This factor has been described as a putative repressor of hGR\(\alpha\) (47–49), but this hypothesis is a subject of debate (50). Furthermore RU486 does not impede the binding of GR to DNA (51, 52) but inhibits the transactivation ability of GR in the presence of dexamethasone. The glucocorticoid receptor(s) expressed in chondrocytes have been poorly studied, and their regulation remains to be examined in further detail in this cell model. Dexamethasone decreases the level and activity of sPLA\(_2\)-IIA mRNA in vascular smooth muscle cells (53), but conflicting data have been reported in mesangial cells (54, 55). Furthermore these data have been obtained in rats, and the rat promoter does not contain any sequence homologous to a GRE in the region corresponding to the human element D. The only study in humans was performed in the hepatoma HepG2 cell line by Haselmann and Goppelt-Struebe (56), who described a partial decrease of oncostatin M-stimulated expression in the presence of dexamethasone. Modulation of sPLA\(_2\)-IIA gene by glucocorticoids are therefore cell- and species-specific and may act at different levels in opposite ways. Regarding the regulation of the sPLA\(_2\)-IIA promoter, we assume that unliganded GRs bind to the 5'-TGTGTTT-3' sequence and increase the basal transcription activity without affecting the relative stimulation by IL-1\(\beta\), as shown by deletions through the element D. The suppression of the IL-1\(\beta\)-induced stimulation of the transcription activity, which was observed by using the \([-326; +20]\) promoter mutated on the \([-231; -224]\) 5'-TGTGTTT-3' sequence, might be due to the activity of inhibitory factors such as those described in HepG2 cells (27). The binding of GR to the element D also explains the stimulation of the promoter activity by dexamethasone, which is abolished by mutation of the 5'-TGTGTTT-3' sequence; the low level of homology with the consensus GRE and the absence of repeated GRE may explain the low level of stimulation.

Mutation of the \([-224; -220]\) 5'-TGCCA-3' CTF/NF1 hemesite suppressed the stimulation of sPLA\(_2\)-IIA transcription activity by dexamethasone without affecting its basal and IL-1\(\beta\)-stimulated levels. The CTF/NF1 family members are encoded by four genes, NF1-A, NF1-B, NF1-C, and NF1-X, and their diversity is increased by alternative splicing or cleavage of larger polypeptides (57–60). The products of the four genes have different transcriptional abilities and generate heterodimers with intermediate activation potentials (61). Stimulations of the human papillomavirus type 16 and aspartate aminotransferase promoters by glucocorticoid require the presence of intact NF1-binding sites (62, 63). The glucocorticoid receptor favors binding of CTF/NF1 factors to the murine mammary tumor virus promoter \textit{in vivo} by stimulating the nucleosome disrupting activity of the SWI/SNF complex (64). In the context of the sPLA\(_2\)-IIA promoter, its main action would be to allow the C/EBP factors to achieve full transcription activities. Such effects are conditioned by interactions between the GR bound to the element D and the C/EBP factors bound to the element C. It is interesting to note that the GR recruits C/EBP\(\beta\) to the rat \(\alpha 1\) acid glycoprotein promoter (65) and that the

**Fig. 10.** Hypothetical model of the interactions between transcription factors bound to the regulatory elements of the human sPLA\(_2\)-IIA promoter, and general transcription factors bound to the TATA box.
downstream GRE is regulated by the upstream C/EBP-binding sites by 53 bp in this promoter (24), the same distance as that between the GR and C/EBP-binding sites in the sPLA2-IIA promoter. Boruk et al. (66) demonstrated that the GR interacts with C/EBPβ through their AF2 domain and an intermediary factor. Chang et al. (67) have suggested that this intermediary factor is, in the context of the α1 acid glycoprotein promoter, the co-activator TIF1β, which belongs to the RING protein family. The identification of the factor(s) ensuring the interaction between the GR and the C/EBP proteins bound to sPLA2-IIA promoter in chondrocytes requires further studies. The hypothesis of the putative regulatory mechanisms of the various factors that bind to human sPLA2-IIA promoter are summarized in Fig. 10.

Increased synthesis of prostaglandin E2 by chondrocytes and synoviocytes in response to IL-1β is a key event of the inflammatory process in joints. Prostaglandin E2 is produced through the functional interaction between cyclooxygenase-2 and sPLA2 in cell types that express both of these genes (68, 69). We have shown in this study that C/EBPβ and C/EBPβ play a critical role in the regulation of the secreted type IIIA sPLA2 promoter activity by IL-1β. We and others have demonstrated that these factors are also essential for the regulation of cyclooxygenase-2 transcription. C/EBPβ and C/EBPβ may therefore be considered as putative targets of therapeutic strategies to inhibit iocosanoid synthesis in chondrocytes.

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