A distal enhancer controls cytokine-dependent human cPLA$_2$α gene expression

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Abstract  Specific control of group IVA cytosolic phospholipase A$_2$, cPLA$_2$α or PLA2G4A, expression modulates arachidonic acid production, thus tightly regulating the downstream effects of pro- and anti-inflammatory eicosanoids. The significance of this pathway in human disease is apparent in a range of pathologies from inflammation to tumorigenesis. While much of the regulation of cPLA$_2$α has focused on posttranslational phosphorylation of the protein, studies on transcriptional regulation of this gene have focused only on proximal promoter regions. We have identified a DNase I hypersensitive site encompassing a 5′ distal enhancer element containing a highly conserved consensus AP-1 site involved in transcriptional activation of cPLA$_2$α by interleukin (IL)-1β. Chromatin immunoprecipitation (ChIP), knockdown, knockout, and overexpression analyses have shown that c-Jun acts both in a negative and positive regulatory role. Transcriptional activation of cPLA$_2$α occurs through the phosphorylation of c-Jun in conjunction with increased association of C/EBPβ with the distal novel enhancer. The association of C/EBPβ with the transcriptional activation complex does not require an obvious DNA binding site. These data provide new and important contributions to the understanding of cPLA$_2$α regulation at the transcriptional level, with implications for eicosanoid metabolism, cellular signaling, and disease pathogenesis.—Bickford, J. S., D. E. Beachy, K. J. Newsom, S. J. Barilovits, J.-D. H. Herlihy, X. Qiu, J. N. Walters, N. Li, and H. S. Nick. A distal enhancer controls cytokine-dependent human cPLA$_2$α gene expression. J. Lipid Res. 2013. 54: 1915–1926.

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As the apex of the eicosanoid pathway, group IVA cytosolic phospholipase A$_2$, cPLA$_2$α or PLA2G4A, is responsible for liberation of arachidonic acid from the sn-2 position of membrane phospholipids, leading to prostaglandin and leukotriene biosynthesis (1, 2). The physiological relevance of this gene is supported by studies in cPLA$_2$α$^{-/-}$ mice, which display age-dependent renal concentrating defects, enlarged hearts, defects in female reproduction (comparable to COX-2$^{-/-}$ mice), and small intestine ulcerative lesions (3, 4). When challenged with lipopoly-saccharide, HCl (5), butylated hydroxytoluene (6), or urethane (7), these mice have attenuated lung inflammation and tumor formation. Most recently, in vitro studies, animal models, and human brain imaging analysis have also linked cPLA$_2$ to the onset and maintenance of Alzheimer’s disease pathogenesis (8).

As the rate-limiting step in eicosanoid production, cPLA$_2$α activity has also been coupled to events controlling signal transduction (9), apoptosis (10), inflammation (11), and cancer (12). The downstream eicosanoids have also been associated with a number of inflammatory diseases, including asthma (13), atherosclerosis, sepsis, Crohn’s disease (14), and rheumatoid arthritis (15), demonstrating the critical roles these bioactive lipids have in normal cellular function and disease pathologies.

It is well documented that the expression and enzymatic activity of cPLA$_2$α are increased by numerous mediators, including interleukin (IL)-1β (15, 16), tumor necrosis factor (TNF)−α, interferon (IFN)−γ (17), macrophage colony stimulating factor (M-CSF) (18), and oncogenic Ras (19), with enzymatic activity requiring calcium increases and protein phosphorylation (4). Wu et al. (20) initially characterized the basal expression of the human cPLA$_2$α promoter with complementary studies on the rat promoter by Tay et al. (21). A number of regulatory factors have been implicated directly and indirectly in the regulation of...
cPLA\(_\alpha\) expression and activity through the proximal promoter, including nuclear factor (NF)-\(\kappa\)B (22, 23), Krüppel-like factor (KLF) (24, 25), hypoxia-inducible factor (HIF-1) (26), and specificity protein 1 (Sp1) (27). A 48 bp CA dinucleotide repeat at approximately −200 bp has been shown to confer a negative regulatory effect on cPLA\(_\alpha\) basal promoter activity (16, 28). Cowan et al. (29) have also implicated an Inv element at the transcriptional start site and a novel TBP binding site at −30 to −35 bp. Nemenoff and coworkers have demonstrated that oncogenic forms of Ras (19) increase transcription of cPLA\(_\alpha\) in normal lung epithelial cells and non-small-cell lung carcinoma (NSCLC) cell lines through proximal promoter elements that bind lung Krüppel-like factor (LKLF) (24) and Sp1 (27) with induction, requiring the activation of the c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) pathways (30). Most recently, Tsou et al. (31) have implicated nucleolin through proximal c-Jun/Sp1 promoter elements in the transcriptional activation of cPLA\(_\alpha\) by phorbol ester.

Chi et al. (32) have very recently argued that a putative AP-1 site in the proximal promoter at approximately −500 bp (TGATTAA), deviating from the consensus AP-1 sequence [TGAG(C/T)CA], is involved in IL-1β-dependent induction of cPLA\(_\alpha\) in rheumatoid arthritis synovial fibroblasts. We and others have clearly demonstrated that the human cPLA\(_\alpha\) promoter (in our studies, extending to approximately −6.8 kb) is not responsive to cytokine activation (21–31). This discrepancy may result from these investigators’ use of diseased synovial fibroblasts in conjunction with IL-1β concentrations 15 times (30 ng/ml) higher than our dosage and well beyond physiologically relevant levels. Liao et al. (33) reported a 1.4-fold response from the proximal promoter, although neither an induction nor basal activity was readily detectable in any of our experiments with the promoter alone.

Given the central importance of cPLA\(_\alpha\) regulation and activity in normal physiology, inflammation, and cancer, we sought to identify a true stimulus-dependent regulatory element responsible for transcriptional regulation of the cPLA\(_\alpha\) gene. In this study, we have characterized a distal DNase I hypersensitive (HS) site that harbors an IL-1β-responsive element. Our results implicate c-Jun as a transcriptional repressor through direct interaction with the enhancer, while transcriptional activation is mediated through association of C/EBPβ with both enhancer and promoter elements.

**MATERIALS AND METHODS**

**Reagents**

FuGENE 6 transfection reagent and complete protease inhibitor cocktail were purchased from Roche Applied Science. IL-1β from R and D Systems, trichostatin A from Calbiochem, restriction enzymes from New England Biolabs, DNase I from Worthington Biochemical, and antibodies from Santa Cruz Biotechnology. The random-primer DNA labeling kit was purchased from Invitrogen, and the Qiagen RNeasy Mini Kit was purchased from Qiagen.

**Cell culture**

Human fetal lung fibroblast (HFL-1) cells or a human bronchoepithelial cell line (S9) (34) were maintained in nutrient mixture F12/Ham Kaighn’s modification (F12K) at pH 7.4 supplemented with 25 mM NaHCO\(_3\), 4 mM glutamine, antibiotic/antimycotic (ABAM) (Gibco) and 10% FBS (Atlanta Biologicals) at 37°C with 5% CO\(_2\), Wild-type (WT) and knockout mouse embryonic fibroblasts (MEF) were grown in DMEM (CellGro) with identical supplements. The C/EBPβ\(\sim\) cell line was provided by Dr. P. Johnson, National Institutes of Health, via Dr. Michael Kilberg (University of Florida), and the c-Jun\(\sim\) and c-Jun overexpressing cell lines (35, 36) were kindly provided by Dr. David A. F. Gillespie (University of Glasgow, United Kingdom).

**DNase I hypersensitive (HS) site and Southern blot analysis**

Ten 150 mm dishes of HFL-1 cells were grown to 75% confluency, then either left untreated or treated for 8 h with IL-1β. Cell permeabilization, DNase I treatment, and Southern analysis were performed as previously described (37, 38). Following DNase I treatment, cell lysates were incubated overnight at 50°C, and genomic DNA was isolated by organic extraction, alcohol precipitation, and resuspension in TE. The DNA samples were digested with BamHI, and 30 μg of each sample were fractionated on a 0.8% HGT agarose gel followed by alkaline denaturation, electrotransfer to a nylon membrane, and UV cross-linking. The membrane was hybridized at 61°C with an end-specific single copy \(^{32}\)P-radiolabeled DNA probe, generated by PCR from a human cPLA\(_\alpha\) genomic clone that indirectly end-labeled the 3’ end of the 18.8 kb BamHI fragment as depicted in Fig. 1.

**DNA isolation, real-time RT-PCR, and northern analysis**

Total RNA was purified using the Qiagen RNeasy Mini Kit with DNase I digestion or by the Chomczynski and Sacchi method with modifications as previously described (39). For real-time PCR analysis, cDNA was generated using a SuperScript First-Strand synthesis kit (Invitrogen) as per the manufacturer’s instructions. Two micro-liters of the resulting cDNA was used for real-time PCR analysis using SYBR Green Supermix with ROX (Bio-Rad) on a Bio-Rad iCycler and analyzed by the ΔΔCT method normalized to cyclophilin A as described previously (40). Crossing threshold (CT) values from both cyclophilin A and respective target genes were used in the ΔΔCT method to calculate relative fold inductions, which were statistically analyzed as described below (40). Real-time primers were as follows: cPLA\(_\alpha\), F: (5’-CGT GAT GTG CCT GTG GTA GC-3’); R: (5’-TCT GGA AAA TCA GGG TGA GAA TAC -3’); cyclophilin A, F: (5’-CAT CCT AAA GCA TAC TGG TCC-3’); R: (5’-GGT CCTT GCC ATT CCT GCT G-3’); human growth hormone, F: (5’-GAACCCCA GAC CTC CTT TGA-3’); R: (5’-CAT TCT CCA GCC TCC CCA T-3’). For northern analysis, 10–20 μg of total RNA was denatured and fractionated on 1% agarose, 6% formaldehyde gel, then electrotransferred to a Zeta-Probe blotting membrane (Bio-Rad) and UV cross-linked. Membranes were hybridized with a gene-specific \(^{32}\)P-radio labeled probe generated by random primer extension (Invitrogen) for human growth hormone (hGH), cPLA\(_\alpha\), or the large ribosomal subunit protein, L7a. The membrane was washed at 65°C and exposed to X-ray film. Densitometry was performed with ImageJ software (http://imagej.nih.gov/ij/). Relative fold inductions were determined as untreated cells compared with IL-1β-treated samples, normalized to the ribosomal protein L7a loading control.

**Generation of promoter and enhancer constructs in human growth hormone reporter vectors**

Various regions of the cPLA\(_\alpha\) promoter were subcloned into the promoterless pUC12-based hGH reporter plasmid p0GH (41).
Fig. 1. IL-1β induction and chromatin structure of cPLA₂ in HFL-1. (A, left) HFL-1 cells were treated with 2 ng/µl IL-1β for the indicated times, and total RNA was analyzed by real-time RT-PCR. Relative fold induction was calculated relative to the untreated (0 h) sample using cyclophilin A as an internal loading control. Crossing threshold (CT) values from both cyclophilin A and mPGES-1 were used in the ΔΔCT method (40) to calculate relative fold inductions. Bars represent the mean of 2 ± SEM (n = 3). *P ≤ 0.05. (A, right) HFL-1 cells were untreated (control) or treated with 2 ng/µl IL-1β for 48 h, and total protein was analyzed by immunoblot analysis with an antibody specific to cPLA₂. (B, top) HFL-1 cells were treated with 1 µM trichostatin A (TSA) for the indicated times, and total RNA was analyzed by real-time RT-PCR. Relative fold expression was calculated relative to the untreated (0 h) sample using cyclophilin A as an internal loading control. Cross-}

Transcriptстроительная структура cPLA₂ в HFL-1. (A, лево) HFL-1 клетки были выращены с 2 ng/µl IL-1β для указанных временных интервалов, и общая РНК была анализирована с помощью реального времени RT-PCR. Относительное умножение индукции рассчитывалось относительно непротравленного (0 h) образца, используя в качестве внутреннего контроля cyclophilin A. Средние значения CT-значений от обеих cyclophilin A и mPGES-1 использовались для расчета относительных умножений. Бары представляют среднее ± SEM (n = 3). *P ≤ 0.05. (A, право) HFL-1 клетки были непротравленными (контроль) или выращены с 2 ng/µl IL-1β за 48 h, и общая протеин была анализирована с помощью иммуноблота с использованием антитела специфического к cPLA₂. (B, верх) HFL-1 клетки были выращены с 1 µM trichostatin A (TSA) за указанные временные интервалы, и общая РНК была анализирована с помощью реального времени RT-PCR. Относительное умножение экспрессии было рассчитано относительно непротравленного (0 h) образца с использованием cyclophilin A как внутреннего контроля. Кривые, соответствующие уровням экспрессии, построены с учетом n = 3. *P ≤ 0.05 и **P ≤ 0.01. (C, верх) Схематическая модель гена cPLA₂ также показывает 18.8 kb BamHI фрагмент с расположением 3′-пробы, используемой для DNase I анализа.

Иммуноблот анализ

Всего протеина (30 µg) от указанных временных точек были выращены на Tris-HCl полиноксиламиновую гель (Bio-Rad) и электротрансферилированы на нитрошлоссовые мембраны (Bio-Rad). Мембранны были затем обработаны 1 h с 8% ненасыщенной сухой клетки в TBST [10 mM Tris-HCl (pH 7.5), 0.1% (v/v) Tween 20, 200 mM NaCl] при комнатной температуре. Мембранны были инкубированы ночью на 4°C с первичными антителами к cPLA₂ (Santa Cruz, sc-4514), p-c-Jun (Santa Cruz, sc-10944), p-c-Jun (Santa Cruz, sc-822), C/EBPβ (Santa

(C, низ) Репрезентативный северный блот иллюстрирует присутствие DNase I HS сайта (левая стрелка, ~9.5 kb) в контроле и IL-1β-протравленных клетках при увеличении концентраций DNase I. Положение DNase I HS сайта указано вверх. Указатель.
Electrophoretic mobility shift assay

Nuclear extracts were obtained by rinsing cells with ice-cold PBS and harvesting in ice-cold PBS with protease inhibitors (Roche). Cells were pelleted by centrifugation at 300 g for 10 min at 4°C, then resuspended in lysis buffer [20 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM MgCl₂, 0.2 M EDTA, 1 mM DTT, 1% Triton X-100, protease inhibitors]. Samples were incubated on ice for 15 min, then centrifuged at 15,000 g for 10 min at 4°C. The supernatant containing nuclear extract was collected, and protein concentration was determined using a BCA assay (Pierce).

Single-stranded oligonucleotides were designed to include the AP-1/CRE-like and consensus AP-1 site in the cPLA₂ enhancer region: sense, 5′-GAATGCCTTGATGACCACTCTCAGTAGTTTGCAT-3′ and antisense, 5′-ATGAAACATAACCAGGAGACCC-3′. The sense strand was left unlabeled (for cold competitor) or biotinylated (for hot-labeled probes). Probes were annealed by mixing complementary oligonucleotides at a 1:1 ratio in annealing buffer [10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl (pH 8.0)] through a temperature gradient. For electrophoretic mobility shift assay (EMSA) reactions, 10 µg of nuclear extract was added to a mixture of binding buffer (9 mM Tris, 45 mM KCl, 100 µM DTT), 2 µg herring sperm DNA, 0.0125% glycerol, and for cold competitor reactions, 0.1 pmol unlabeled double-stranded DNA probe, then incubated at room temperature for 5 min. Biotinylated double-stranded DNA probes (0.5 fmol) were added, followed by incubation at room temperature for 20 min. Samples were run on a 5% Tris-Glycine gel (Bio-Rad) at 200 V, then transferred to a nitrocellulose membrane. Membranes were probed for biotin-labeled DNA-protein complexes using a LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

DNA affinity purification assay

For DNA affinity purification assay (DAPA) (42), 5 µg of biotinylated DNA double-stranded probe and 500 µg nuclear extract (described above) from HFL-1 cells, 89 cells, or mouse fibroblasts was incubated with 4% streptavidin-agarose beads (Sigma). The samples were rotated at room temperature for 1 h, and then centrifuged at 1,000 g for 1 min. The supernatant was removed, and the pellet was washed four times with 1 ml ice-cold PBS, then resuspended in 100 µl SDS loading buffer. Samples were boiled for 5 min and run on a 10% Tris-HCl gel (Bio-Rad). Immunoblot analysis for c-Jun was performed as described.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as previously described with an average chromatin sonication size of ~500 bp (43). Briefly, HFL-1 cells were treated with IL-1β for 8 h followed by protein-DNA crosslinking with 1% formaldehyde for 10 min. Cell extracts were incubated overnight at 4°C with 2 µg of control rabbit IgG (sc-2027) or the following rabbit antibodies: c-Jun (sc-1694), C/EBPβ (sc-150); RNA Pol II (sc-899), p300 (sc-584), or Sp1 (sc-59). All antibodies for ChIP were purchased from Santa Cruz. Purified DNA was used for real-time PCR analysis. We analyzed our data relative to the fraction of total isolated chromosomal DNA prior to immunoprecipitation (input) rather than to IgG. The data were analyzed by two-way ANOVA followed by Tukey’s post hoc test. We strongly feel that presenting the data relative to input is more reflective of any actual changes. The following primers were used: enhancer region, F: (5′-AGG GCA GAT GTT TCT CAG CG-3′); R: (5′-GCC ATT TTA TTT TAT GTG TAT GTT CTT-3′); promoter region, F: (5′-CAA ACT CCT GTT TCT AAT AAC TAA GCA-3′); R: (5′-TTG CTG ACA GTT CCC AGA GTT ACC-3′).

Immunoprecipitation

Due to the low sensitivity of the available serine 63-phosphorylated c-Jun (Santa Cruz, sc-822), c-Jun was immunoprecipitated prior to immunoblot analysis. HFL cells were treated with IL-1β for 4 or 8 h. Cells were collected in PBS, lysed with RIPA lysis buffer [150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl (pH 7.5)] and incubated at 4°C overnight with an antibody against c-Jun (Santa Cruz, sc-1694). Complex capture was completed by incubating with Protein A Sepharose beads at 4°C for 2 h. Complexes were washed four times with RIPA buffer, followed by immunoblot analysis with an antibody against serine 63-phosphorylated c-Jun (Santa Cruz, sc-822).

siRNA knockdown

HFL-1 cells at 40% confluence were transfected with a final concentration of 100 nM Dharmacon SMARTpool siRNA for c-Jun or C/EBPβ using DharmaFECT 1 siRNA transfection reagent. After 72 h, cells were collected and immunoblot analysis was used to assess protein knockdown, followed by real-time RT-PCR for cPLA₂ gene expression using primers described above.

Statistical analysis

Data points are the means from at least three independent experiments, and error bars represent the standard error of the mean (SEM). Statistical analyses were determined by paired or unpaired Student t-test. P ≤ 0.05 was considered statistically significant.

RESULTS

Induction of cPLA₂α and detection of DNase I hypersensitive sites in the cPLA₂α gene locus

To establish the induction of cPLA₂α in our cells, HFL-1 cells were treated with IL-1β for both RNA and protein analyses. Following 4, 6, or 8 h of IL-1β treatment, cPLA₂α mRNA was significantly induced in these cells as determined by real-time RT-PCR. cPLA₂α protein levels were elevated by 48 h of stimulation as shown by immunoblot analysis (Fig. 1A). It has been previously shown that induction of cPLA₂α gene expression by the proinflammatory cytokines IL-1β, TNFα and IFN-γ requires de novo transcription (16, 44). To date, limited information exists regarding regulatory elements that mediate cytokine-dependent induction of cPLA₂α, with most data focusing on only basal expression from the proximal promoter (16, 24, 27, 32).

The state of chromatin acetylation is known to contribute to the chromatin state and the regulation of gene expression. Therefore, we treated HFL-1 cells with a histone deacetylase inhibitor, trichostatin A (TSA), to determine
whether increased acetylation would lead to induction of the cPLA<sub>α</sub> gene. As shown by northern blot analysis and corresponding densitometry (Fig. 1B), TSA was able to induce cPLA<sub>α</sub> in a time-dependent manner, indicating that cPLA<sub>α</sub> gene induction may be linked to changes in histone acetylation and potential alterations in chromatin structure. We first analyzed a 4.1 kb cPLA<sub>α</sub> promoter fragment in a reporter construct by transient transfection and found this fragment to be unresponsive to IL-1β (shown in Fig. 2A). Based on our prior experience with cytokine-dependent intronic enhancers elements, we also tested a series of fragments spanning the cPLA<sub>α</sub> gene locus and found them to also be unresponsive to IL-1β (supplementary Fig. 1). Therefore, to identify potential distal upstream regulatory regions, DNase I HS analysis was employed to detect any open chromatin structure. Control or IL-1β-treated HFL-1 cells were exposed to increasing concentrations of DNase I. The data from Southern analysis (Fig. 1) illustrate a constitutive DNase I HS site at 14 kb upstream of the transcriptional start site. We have previously shown that constitutive HS sites harbor specific inducible elements (37, 45); therefore, we chose to further analyze this region.

### Identification of a distal IL-1β-responsive enhancer element

To test the functional relevance of the 5′ distal HS site, a series of promoter fragments were analyzed in a hGH reporter plasmid (Fig. 2A, top) transiently transfected into HFL-1 cells and evaluated by northern analysis. The −14 kb promoter fragment, which harbors the HS site, was the only construct that displayed either basal or IL-1β responsiveness (−2-fold) (Fig. 2A, bottom).

To further characterize this region, a 2.3 kb fragment flanking the HS site (Fig. 2A) was subcloned from the −14.0 kb promoter into a hGH reporter construct containing either a minimal −1.4 kb cPLA<sub>α</sub> promoter (Fig. 2A) or a heterologous minimal viral TK promoter (Fig. 2B). Similar to the −14.0 kb promoter, this 2.3 kb fragment, in either the forward (2.3F) or reverse (2.3R) orientation, displays both basal activity and IL-1β responsiveness, indicative of a stimulus-specific enhancer element. The enhancer activity within the 2.3 kb fragment was further analyzed with a series of internal deletions (Fig. 3). First, the 3′ end of the 2.3 kb fragment was deleted, creating a 1.6 kb fragment that retains enhancer activity (Fig. 3A). Subsequent internal deletions of the 2.3 kb (IV) and 1.6 kb (I, II, III and V) were similarly illustrated and analyzed in Fig. 3B. These studies led to the identification of a minimal enhancer region (Fig. 3A, gray box) based on the complete lack of basal and enhancer activity in fragments I, II, and IV, which lack this region, compared with the inclusion of this region in fragments 2.3 and 1.6 kb, III, and V, which display both basal and induced expression (Fig. 3A, B).

### Identification of c-Jun as a cognate factor binding to the cPLA<sub>α</sub> enhancer

The sequence of the minimal enhancer region spanning from −9654 to −9340 (315 bp) of the cPLA<sub>α</sub> 5′ flanking region was analyzed using the TESS software (www.cbil.upenn.edu/cgi-bin/tess/tess). We identified a consensus AP-1 site (TGAGTCA, gray shading) (Fig. 4A).
The selective deletion of either a control upstream (control A) or downstream (control B) site relative to the AP-1 sequence had no effect on expression levels (Fig. 4A, B). On the other hand, the selective deletion of the AP-1 consensus site (Fig. 4A, bold letters) caused a striking loss of both basal and IL-1β-dependent enhancer activity, as shown in a representative northern analysis (Fig. 4B, right). The importance of this AP-1 sequence is further demonstrated by a strikingly high level of cross-species conservation illustrated by the alignment provided in supplementary Fig. II. This alignment also provides a strong argument for the critical importance of this AP-1 site and the homology found in the surrounding sequences.

To analyze the relevance of this particular site in vitro, we utilized a biotinylated oligonucleotide flanking the AP-1 site in an EMSA (Fig. 4C, top); we identified a shifted complex from HFL-1 nuclear extracts that was inhibited by unlabeled cold competitor. To demonstrate that this complex contained c-Jun, we utilized DAPA (42) to analyze complexes using the same oligonucleotide binding site with nuclear extracts from both HFL-1 cells and a human bronchopulmonary epithelial cell line, S9, specifically demonstrating reactivity with a c-Jun antibody by immunoblot analysis (Fig. 4C, bottom).

To determine the in vivo response of this element to c-Jun, the aforementioned constructs were cotransfected along with the prominent AP-1 family member c-Jun. Overexpression of human c-Jun induces reporter gene expression when the 2.3 kb cPLA2α enhancer fragment is coupled to either the cPLA2α (1.4 kb) or the minimal TK promoter (Fig. 4D). Furthermore, we also observed an increase in reporter activity from fragment III (Fig. 3B) cloned in front of the TK promoter in response to c-Jun overexpression (Fig. 4D). These data demonstrate that c-Jun can function to induce cPLA2α gene expression through the identified enhancer region, which includes the highly conserved AP-1 site (supplementary Fig. II).

Having linked c-Jun with a specific binding site within the enhancer, we then sought to determine the effect of IL-1β on c-Jun abundance and phosphorylation. IL-1β treatment did not affect the levels of c-Jun protein in HFL-1 cells based on immunoblot analysis (Fig. 4E, top). It is well documented, however, that phosphorylation of c-Jun at serine 63 is required for both gene derepression (46–49) and activation (50, 51); therefore, we evaluated the effect of IL-1β on c-Jun phosphorylation at S63. Due to the low affinity of the available phospho-serine 63 antibody, total c-Jun was first immunoprecipitated to obtain sufficient proteins levels for detection by immunoblot analysis with this antibody. We observed a time-dependent increase in c-Jun phosphorylation at serine 63 following IL-1β treatment, consistent with the role of c-Jun in derepression and activation (Fig. 4E, bottom).

Analysis of the enhancer and promoter regions by ChIP

Having implied that c-Jun is capable of interacting with the putative AP-1 site in vitro by EMSA and DAPA analyses (Fig. 4C), we next addressed the association of c-Jun with the cPLA2α enhancer element in situ by ChIP analysis. ChIP analysis of c-Jun showed a constitutive association with the enhancer (Fig. 5A), whereas we were unable to identify c-Jun at the promoter. Although IL-1β does not elicit a change in the cellular levels (Fig. 4E, top) or abundance of c-Jun at the enhancer (Fig. 5A), IL-1β does cause a time-dependent increase in c-Jun phosphorylation at S63, linking this posttranslation modification with the role of this transcription factor in cPLA2α gene expression. Due to the low affinity of the S63 phospho-c-Jun antibody, some of our ChIP results for S63 phospho-c-Jun demonstrated occupancy at the enhancer, whereas others did not (data not shown). Therefore, in conjunction with increased S63 phosphorylation in response to IL-1β (Fig. 4E, bottom), we believe that it is likely that a fraction of c-Jun at the enhancer is similarly phosphorylated, consistent with its role in derepression and activation.
Defining a cytokine-dependent cPLA2α enhancer element

**Fig. 4.** Characterization of the cPLA2α enhancer. (A) Sequence of fragment III (Fig. 2C) containing the enhancer region from 9654 to 9340 bp upstream of the transcription start site. The consensus AP-1 (TGAGTCA, gray box) is shown as underlined italicized text. The black bold text indicates the nucleotides at the AP-1 site and the control regions that were deleted for analysis in (B). (B) Representative northern blots illustrate the constructs containing the TK promoter with intact region III (Fig. 3B) or the site-specific deletions (A) with or without IL-1β. Resulting membranes were probed for hGH and L7a. (C, top) A representative autoradiogram of an EMSA using a fragment containing the indicated AP-1 site depicts a shifted complex in the presence of nuclear extract (NE) isolated from HFL-1 cells. A cold unlabeled competitor was used to demonstrate specific interaction. (C, bottom) A representative autoradiogram of a DAPA shows the specific interaction of c-Jun with the fragment used above with extracts from HFL-1 cells, human bronchoepithelial cells (S9) with or without c-Jun overexpression, and a negative mouse fibroblast cell line. The resulting membrane was probed with an antibody specific to c-Jun. (D) An enhancer fragment, 2.3 kb or fragment III, was cloned in front of either the endogenous 1.4 kb cPLA2α promoter or the TK promoter with or without c-Jun overexpression, and resulted in the phosphorylation of c-Jun at serine-63.

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We also evaluated C/EBPβ by ChIP analysis because of its involvement in transcriptional activation of other members of the eicosanoid pathway, specifically cyclooxygenase (COX)-2 and microsomal prostaglandin E synthase (mPGES), based on our studies and others (38, 52). We found that both c-Jun and C/EBPβ were constitutively associated with the enhancer in control cells and that the association of C/EBPβ increased more than 2-fold following 8 h of IL-1β treatment (Fig. 5A). Similarly, we observed a ~2-fold IL-1β-dependent increase in RNA polymerase II (Pol II) association with the enhancer along with a low level of interaction with p300, while IgG and Sp1 served as negative controls. We next evaluated the occupancy of these factors at the promoter region and found that the association of Pol II and C/EBPβ with this region was increased after 8 h of cytokine treatment (Fig. 5B), with a small but significant association of p300 and Sp1 compared with IgG, as previously documented (23, 31). Pol II was also found inductively associated with the promoter at 4 h of cytokine exposure (Fig. 5B, inset), as would be expected given the induction of cPLA2α mRNA by IL-1β at 4 h (Fig. 1A). Of note, our enhancer fragment, which contained all the necessary sequences for IL-1β-dependent induction (Fig. 4), lacked any identifiable C/EBPβ consensus-binding sequence. Similarly, we also searched within ~500 bp of the promoter region sampled by our ChIP analysis and found no C/EBPβ binding sites. Together these results indicate that C/EBPβ associated with these regions independent of direct DNA binding.

**Functional role of C/EBPβ and c-Jun on cPLA2α gene expression**

To address the functional roles of C/EBPβ and c-Jun, HFL-1 cells were transfected with specific siRNA pools for each gene. Immunoblot analysis demonstrated equivalent knockdown of C/EBPβ and c-Jun by their respective siRNAs (Fig. 6A, left). IL-1β-dependent induction of cPLA2α mRNA levels at 8 h was inhibited more than 50% by C/EBPβ siRNA, whereas c-Jun knockdown showed no effect (Fig. 6A, right) despite substantial knockdown of c-Jun protein levels. A potential explanation for the ineffectiveness of c-Jun siRNA on cPLA2α induction may be due to redundancy among the large AP-1 family (containing members from the Jun, Fos, and ATF subfamilies). We therefore evaluated the effects of overexpression of various AP-1 family members (JunB, JunD, FosB, c-Fos, and ATF2) on enhancer fragment III coupled to either the TK or endogenous promoter (supplementary Fig. III). All of these AP-1 family members were able to induce either promoter/enhancer construct to varying degrees, demonstrating redundancy and thus explaining the inability of c-Jun knockdown to affect cPLA2α induction. In addition, it would appear that any member of the AP-1 family can replace c-Jun at this site, thus providing no obvious route for analysis of occupancy by any specific member of this large family.

To further establish the functional importance of C/EBPβ and c-Jun with respect to IL-1β induction of cPLA2α, we evaluated the cytokine response in two separate WT MEF cell lines and MEFs derived from C/EBPβ-/- (53) and c-Jun-/- mice (35, 36). While siRNA provided a significant decrease in protein levels for each factor (Fig. 6A, left), the use of knockout MEFs allowed for complete gene ablation. Real-time RT-PCR of total RNA in control or IL-1β-treated cells for 8 h demonstrated an identical and statistically significant increase in cPLA2α mRNA levels in both WT MEF cell lines (Fig. 6B). Consistent with the siRNA results (Fig. 6A, right), we observed no increase in cPLA2α mRNA expression in response to IL-1β exposure in the C/EBPβ-/- cells compared with the induction in WT MEFs (Fig. 6B). The combination of ChIP,

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**Fig. 5.** Identification of transcription factors associated with the cPLA2α enhancer fragment. HFL cells were treated with IL-1β for the indicated times, followed by ChIP analysis as described in Materials and Methods. (A) ChIP analysis was performed at 8 h using primers specific to the cPLA2α enhancer region with the indicated antibody. Bars represent the signal relative to the total input ± SEM as determined by real-time PCR (n ≥ 3). (B) ChIP analysis was performed at 8 h or 4 h (inset) using primers specific to the cPLA2α promoter region with the indicated antibody. Bars represent the signal relative to the total input ± SEM as determined by real-time PCR (n ≥ 3). *P ≤ 0.05 and †P ≤ 0.05 compared with control (gray bar) or IgG, respectively.
knockdown, and knockout studies demonstrate the critical involvement of C/EBPβ in the IL-1β-dependent induction of cPLA₂α, through a mechanism independent of DNA binding.

Conversely, we observed a higher level of induction of cPLA₂α mRNA levels following IL-1β treatment in the c-Jun⁻/⁻ MEFs compared with both WT MEF cell lines. We believe that the higher level of induction in the c-Jun⁻/⁻ MEFs resulted from both the lack of repressive effects of unphosphorylated c-Jun and the redundancy afforded by other AP-1 family members (supplementary Fig. III). Consistent with this observation, stable high-level overexpression of c-Jun in the c-Jun⁻/⁻ cells prevented the IL-1β-dependent induction compared with the WT MEFs or the c-Jun⁺/⁺ cells (Fig. 6B). This may result from the inability of endogenous kinase to phosphorylate overexpressed levels of c-Jun, with S63 phospho-c-Jun being a prerequisite for derepression. The c-Jun⁻/⁻ cell lines were kindly provided by the laboratory of Dr. David Gillespie, who also verified the levels of c-Jun in these cell lines by immunoblot analysis (35, 36).

As C/EBPβ-overexpressing MEFs were unavailable, we transiently overexpressed the full-length isoform of C/EBPβ in S9 cells to evaluate endogenous cPLA₂α gene expression. We utilized the human bronchoepithelial cell line S9, as we have found that this cell line can be more efficiently transfected by plasmid, which is the only manner by which we can observe effects on endogenous gene expression. As shown (Fig. 6C), C/EBPβ expression is capable of causing a greater than 2.5-fold increase in endogenous cPLA₂α levels.

**DISCUSSION**

Many studies have established a direct link between cPLA₂α activity and a wide variety of physiological and pathological events with critical roles in apoptosis, inflammation, and cancer (54, 55). The regulation of cPLA₂α has previously focused on posttranslational, site-specific phosphorylation events; however, numerous studies have documented events associated with transcriptional regulation (4). Until now, all studies have focused solely on the proximal promoter region where, in our hands, sequences extending as far as 6.8 kb upstream of the transcriptional initiation site show no response to cytokines in lung cells. In this study, DNase I HS site analysis (Fig. 1) has led to the discovery of a 5′ distal IL-1β-responsive cPLA₂α enhancer element at approximately −9.5 kb (Figs. 2–4). Our data demonstrate that the enhancer is required for and contributes to both basal and IL-1β-dependent gene expression. We believe that our results provide functionally and physiologically relevant data identifying a cytokine-responsive enhancer element for the human cPLA₂α gene.

A perfect consensus AP-1 site within the enhancer is required for both basal and induced levels of reporter gene expression based on deletion analysis (Fig. 4A, B). This AP-1 site and the surrounding DNA sequences are strikingly conserved across a highly diverse range of mammals (supplementary Fig. II), further substantiating the importance of this AP-1 site. We demonstrated that c-Jun binds to the AP-1 site based on both in vitro EMSA and DAPA analyses (Fig. 4C). ChIP analysis further demonstrated the constitutive association of c-Jun in vivo with the enhancer in both basal and induced cells (Fig. 5A). A transiently transfected reporter construct containing the enhancer was transcriptionally activated in response to c-Jun overexpression (Fig. 4D). IL-1β did not affect the occupancy of c-Jun at the enhancer nor did it alter cellular levels of c-Jun. However, we did observe a time-dependent increase of c-Jun phosphorylation at serine 63 of the transactivation domain in response to IL-1β (Fig. 5C). The phosphorylation of this serine residue has been strongly associated with c-Jun activation (56). We would argue that phosphorylation of c-Jun and its cognate AP-1 site are clearly critical to cPLA₂α induction by cytokines.

Regarding c-Jun occupancy, as shown at the enhancer region by ChIP analysis in Fig. 5A, c-Jun was always present at the enhancer in control and IL-1β-treated cells, and we saw no c-Jun binding at the promoter. We believe, based on the knockdown data in Fig. 6B showing that cPLA₂α expression was significantly increased in c-Jun⁻/⁻ cells, that c-Jun is part of a repressive complex in the basal state on the endogenous gene. Our data support a mechanism where IL-1β-dependent phosphorylation of endogenous c-Jun at serine 63 (Fig. 4E) causes the dissociation of a repressive complex allowing phospho-c-Jun to participate in gene activation. c-Jun’s role as an activator is supported by the ability of c-Jun overexpression to cause transcriptional activation through the enhancer coupled to either the TK or 1.4 kb cPLA₂α promoter (Fig. 4D). In addition, we have shown that numerous members of the AP-1 family when overexpressed can induce reporter gene expression through the enhancer coupled to either of these promoters (supplementary Fig. III). Therefore, c-Jun can function as a repressor in the basal state and participate in the cytokine-dependent induction, while the other AP-1 family members can function as activators alone or redundantly in the absence of c-Jun (Fig. 6B).

C-Jun’s role in a repressive complex is substantiated by similar results in numerous other genes (46–49). In fact, recent studies have demonstrated that the repressive complex interacts with c-Jun to repress gene expression (50, 51). Ogawa et al. (51) have shown that repressor complexes on AP-1 target genes specifically require the presence of c-Jun, which is consistent with our ChIP data demonstrating constitutive occupancy of c-Jun at the enhancer. In addition, these investigators have demonstrated that the role of the corepressor complex is to block the exchange of c-Jun for active c-Jun/AP-1 heterodimers, which is also consistent with our data on the ability and the other AP-1 family members to induce cPLA₂α (supplementary Fig. III). Furthermore, these studies have shown that the release of the repressor complex occurs specifically in response to c-Jun phosphorylation at serine 63, consistent with our data demonstrating IL-1β-dependent phosphorylation of c-Jun at serine 63 (Fig. 4E). Therefore, we have been able to demonstrate that c-Jun can function both to maintain cPLA₂α in the off state and to serve as an activator upon cytokine stimulation. The function of c-Jun as a
For cPLA2-lated RNA was used for real-time RT-PCR for cPLA2.

C/EBP 

0.05 compared with IL-1

bodies specific for c-Jun or C/EBP protein was subjected to immunoblot analysis and probed with anti-

Bars represent mean ± SEM (n = 3); *P ≤ 0.05 compared with IL-1β-treated WT cells (black bar); †P ≤ 0.05 compared with IL-1β-treated WT cells (gray bar).

Fig. 6. Effects of knockdown, knockout, and overexpression of c-Jun and C/EBPβ on endogenous cPLA2α expression. (A) HFL-1 cells were transfected with the indicated siRNA. (A, left) Isolated protein was subjected to immunoblot analysis and probed with antibodies specific for c-Jun or C/EBPβ. (A, right) Isolated RNA from siRNA and IL-1β-treated HFL-1 cells was used for real-time RT-PCR for cPLA2α expression. Bars represent mean 2ΔΔCT ± SEM (n = 3).

(B) Total RNA from two wild-type MEF cell lines (WT I and WT II), C/EBPβ−/− MEF, c-Jun−/− MEF, and c-Jun+/− MEFS overexpressing c-Jun (c-Jun+) with or without IL-1β treatment was analyzed by real-time RT-PCR for cPLA2α expression. Bars represent mean 2ΔΔCT ± SEM (n ≥ 3); *P ≤ 0.05 compared with control cells (gray bar); †P ≤ 0.05 compared with IL-1β-treated WT cells (black bar).

(C) S9 human bronchoepithelial cells were transfected with a pcDNA3.1 expression plasmid containing the full-length isoform of human C/EBPβ. Isolated RNA was used for real-time RT-PCR for cPLA2α expression. Bars represent mean 2ΔΔCT ± SEM (n = 3); *P ≤ 0.05.

repressor in the basal state is consistent with the extremely low levels of basal expression of cPLA2α that we observed, which is reasonable considering the potentially detrimental impact of high levels of phospholipase expression on normal membrane integrity.

Our ChIP data demonstrated association of C/EBPβ with both the enhancer and promoter regions (Fig. 5A, B). Most importantly, its levels increased at both sites following cytokine stimulation. Overexpression of C/EBPβ caused a greater than 2.5-fold increase in endogenous cPLA2α mRNA levels (Fig. 6C). Furthermore, functional studies using either siRNA knockdown or knockout cell lines demonstrated that C/EBPβ is a transcriptional activator necessary for IL-1β-dependent induction (Fig. 6A, B). These data strongly establish the importance of this transcription factor for cPLA2α gene expression. The literature clearly demonstrates that C/EBPβ/p300 complexes are recruited to regulatory elements, thus facilitating assembly of the preinitiation complex (PIC) (57, 58). This is consistent with our data, which show an inducible association of Pol II with the distal enhancer and proximal promoter regions as well as an association of p300 with the enhancer. We searched the region surrounding the enhancer that was tested by our ChIP analysis (within approximately 500–1000 bp based on chromatin sonication and primer location), but we did not identify an acceptable binding site. Thus, we believe that C/EBPβ is not directly binding DNA because no consensus sequence for C/EBPβ exists within the fragments that have enhancer activity. In addition, our deletion of the AP-1 site completely abolished cytokine induction; therefore, we believe that C/EBPβ is an activator of cPLA2α and that its activity is mediated through interaction with the transcription complex (possibly p300, which it is known to interact with) independent of DNA binding. This is consistent with several studies that demonstrate that C/EBPβ and other C/EBPs can exert transcriptional effects independent of direct DNA binding (59–62). This is not an uncommon transcriptional regulatory theme in that steroid receptors (63–65), the aryl hydrocarbon receptor (AhR) (66), FOXA1 (67), and the thyroid hormone receptor (68) can all function to regulate gene expression through protein-protein interactions independent of direct DNA binding. Analogous to our results on C/EBPβ, ChIP analysis has shown that the PHOX2A transcription factor can also associate with and regulate the human α3 nicotinic receptor subunit promoter through a DNA-independent mechanism (69). Furthermore, genomewide ChIP-chip arrays and ChIP-seq data for AhR indicates that ∼50% of the AhR-enriched regions lack any dioxin response element (DRE) (70). We therefore believe that C/EBPβ functions as a transcriptional activator through interaction with a large transcription complex, including p300, independent of DNA binding.

In summary, in the basal state c-Jun is bound to the enhancer as a repressor while C/EBPβ is associated with both the promoter and enhancer regions. Upon cytokine stimulation, c-Jun becomes phosphorylated, which allows it to function as an activator, and additional C/EBPβ is recruited to both regulatory regions. Our data identify the first documented cytokine-dependent enhancer element for the human cPLA2α gene and the critical role of c-Jun...
and C/EBPβ. It is interesting that both c-Jun and C/EBPβ have been found to regulate COX-2, the subsequent enzyme in the eicosanoid pathway (71, 72). Analogously, our previous studies established C/EBPβ in the cytokine-dependent regulation of mPGES-1 (38), a terminal synthase in the prostaglandin arm of the eicosanoid pathway. As these are the first results to address the cytokine-dependent regulation of cPLA2α, the role of these cognate transcription factors may have important implications in the overall regulation of eicosanoid biosynthesis.

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