Regulation of human microsomal prostaglandin E synthase-1 by IL-1β requires a distal enhancer element with a unique role for C/EBPβ

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INTRODUCTION

Prostanoids are potent mediators of inflammation, vasodilatation, bronchoconstriction, parturition, sleep cycle regulation and a host of diseases [1–5]. These eicosanoids are commonly derived from arachidonic acid liberated from membrane phospholipids by the phospholipase A2 enzymes [6]. PG (prostaglandin) endoperoxide H synthase, also known as COX (cyclo-oxygenase)-1 and -2, metabolizes arachidone to PGG2 and finally PGH2. PGH2 serves as the substrate for the terminal synthases, producing PGD2, PGE2, PFα2, TxA2 (thromboxane A2) [7,8]. PGE2 has a role in implantation/parturition, arthritis, pyresis, sleep–wake cycle, atherosclerosis, tumorigenesis, pain, kidney function, inflammatory bowel disease, bronchoconstriction and cytoprotective effects in the lung [9–13]. Controlling PGE2 biosynthesis has become an increasingly important therapeutic target in human physiology and pathology [14]. Most relevantly, recent drug discovery initiatives have been redirected to the terminal PGE2 synthases, especially mPGES-1 (microsomal PGE synthase 1), as the regulated source of PGE2 [15–17].

To date, five enzymes with PGE2 synthase activity have been identified, including two membrane or microsomal PGE synthases (mPGES-1 and -2), a cytosolic PGE synthase (cPGES-1/p23) and two Mu class GSTs (glutathione transferases) (GSTM2-2 and GSTM3-3) [18–21]. Whereas cPGES-1 and mPGES-2 are constitutively expressed, mPGES-1 is inducibly expressed, and numerous studies have shown that although all forms of PGE2 contribute to the production of PGE2, mPGES-1, like COX-2, is strongly up-regulated in response to pro-inflammatory stimuli [22,23] and inhibited by glucocorticoids. mPGES-1 has been shown to be the major producer of PGE2 [7,14,24] with high endogenous expression in cancer cell lines [25,26].

The importance of PGE2 in the inflammatory response has been highlighted by mPGES-1−/− mice. Studies utilizing mPGES-1−/− mice have demonstrated beneficial consequences in models of endotoxin-induced shock, arthritis, fever, pain, stroke and anorexia [27–32]. For example, LPS (lipopolysaccharide)-induced PGE2 levels appear to be significantly decreased in mPGES-1−/− mice, demonstrating the importance of mPGES-1 expression/activity [27,33]. In addition, deletion of the mPGES-1 gene (PTGES) also affects the metabolism of PGH2, by altering the prostanoic profile which, in mPGES-1−/− mice, is TxB2 (thromboxane B2)>6-keto-PGFα2 (PGI2 metabolite)>PGFα2>PGE2 compared with PGE2>TxB2>6-keto-PGFα2>PGFα2>PGD2 in WT (wild-type) animals [28,31].

The transcriptional regulation of mPGES-1 has been attributed to a proximal promoter region highlighted by two GC boxes mediated through an interaction with Egr-1 (early growth-response factor 1) [26]. Numerous laboratories have also demonstrated that either Egr-1 overexpression or cytokine treatment results in a 2–3-fold induction of a minimal mPGES-1 promoter [34–38]. Further studies using Egr-1 siRNA (small interfering RNA) in lung adenocarcinoma cells or synovial

*Abbreviations used: C/EBPβ, CCAAT/enhancer-binding protein β; ChIP, chromatin immunoprecipitation; COX, cyclo-oxygenase; cPGES, cytosolic prostaglandin E synthase; Egr-1, early growth-response factor 1; HFL-1, human fetal lung fibroblast; hGH, human growth hormone; hhnRNA, heterogeneous nuclear RNA; HS, DNase I-hypersensitive site; IL-1β, interleukin 1β; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblast; mPGES-1, microsomal prostaglandin E synthase 1; PG, prostaglandin; RT, reverse transcription; siRNA, small interfering RNA; TBST, Tris-buffered saline with Tween 20; TK, thymidine kinase; TxB2, thromboxane B2; UTR, untranslated region; WT, wild-type.

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fibroblasts revealed that inhibition of Egr-1 can partly reduce induction through a minimal mPGES-1 promoter–reporter construct [36,39]. Most importantly, the current studies and others show that cytokines cause an 8–9-fold induction of endogenous mPGES-1 mRNA expression, whereas all studies agree that the proximal promoter accounts for only a 2–3-fold induction. We therefore hypothesized that, apart from the proximal promoter harbouring a potentially important Egr-1 site, there must be other cis-acting regulatory elements required to recapitulate the full cytokine-dependent induction of mPGES-1 expression. Our results demonstrate the identification and characterization of a novel IL-1β (interleukin 1β)-responsive distal enhancer element controlled through C/EBPβ (CCAAT/enhancer-binding protein β) which contributes to the induction of endogenous mPGES-1.

EXPERIMENTAL

Reagents

FuGENE® 6 transfection reagent and Complete™ protease inhibitor cocktail were purchased from Roche Applied Science, IL-1β was from R&D Systems, restriction enzymes were from New England Biolabs, L-α-lyso-lecithin was from Calbiochem, DNase I was from Worthington Biochemical and anti-mPGES-1 monoclonal and polyclonal antibodies were from Cayman Chemical. Monoclonal and polyclonal antibodies against C/EBPβ and Egr-1, as well as an antibody against a polystyrene domain, were purchased from Santa Cruz Biotechnology. The random-primer DNA-labelling kit was purchased from Invitrogen and the RNeasy Mini Kit was purchased from Qiagen.

Cell culture

HFL-1 (human fetal lung fibroblast) and rat pulmonary epithelial (L2) cells from the A.T.C.C. (Manassas, VA, U.S.A.) were grown in Ham’s F12K medium (Sigma) and MEFs (mouse embryonic fibroblasts) (WT and C/EBPβ−/−), provided by Dr P. Johnson (National Institutes of Health, Bethesda, MD, U.S.A.), via Dr Michael Kilberg (University of Florida), were grown in DMEM (Dulbecco’s modified Eagle’s medium) (CellGro). All media were supplemented with 10% (v/v) fetal bovine serum, ABAM (10 μg/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B) and 4 mM glutamine at 37°C in humidified air with 5% CO2.

RNA isolation, Northern blot analysis and hybridization

Total cellular RNA was isolated as described by Chomczynski and Sacchi [40] with modifications [41]. Briefly, following phenol extraction, the RNA was precipitated in 2 M LiCl and washed with ethanol. A 20 μg amount of total RNA was size-fractionated on a 1% agarose/formaldehyde gel as described previously [41]. The RNA was electrophoresed on to a nylon membrane (Zeta-Probe Membrane, Bio-Rad Laboratories) and UV-cross-linked. The membrane was hybridized overnight with the following random-primed double-stranded 32P-labelled gene-specific probes: human mPGES-1 at 65°C, hGH (human growth hormone) or human large ribosomal subunit L7a, each at 61°C. The membranes were then washed with a high-stringency buffer at 65°C and analysed by autoradiography. As indicated, total cellular RNA was also isolated using the RNeasy Mini Kit and analysed by real-time RT (reverse transcription)–PCR. Densitometry was quantified from autoradiography films and analysed with ImageJ software (NIH). The relative fold induction was determined following normalization to the L7a internal control.

First-strand DNA synthesis and real-time PCR

A 1 μg amount of total RNA was used to generate first-strand cDNA (SuperScript® first-strand synthesis kit, Invitrogen) and diluted 5-fold. Real-time PCR was conducted using 2 μl of cDNA as the template, 0.3 μM of each primer, 12.5 μl of iTag SYBR® Green Supermix with ROX (Bio-Rad Laboratories) and water to a final volume of 25 μl. The primers used for amplification are as follows: human mPGES-1 mRNA, 5′-GCCGCCGTGGCTATACC-3′ (forward) and 5′-GGTTGTGACGAATTTCCTTACC-3′ (reverse); hGH, 5′-GAAACCCAGACCTCCTCCT-3′ (forward) and 5′-CATCTCCAGCTCCTCCCAT-3′ (reverse); human mPGES-1-hnRNA (heterogeneous nuclear RNA), 5′-TGGCCTGTAATGGGTAGTG-3′ (forward) and 5′-AGGAAAGGGGTATGGG-3′ (reverse); mouse mPGES-1, 5′-TAGAGGTGGCAGGTCAGAG-3′ (reverse) and 5′-CATCTCCAGCTCCTCCCAT-3′ (reverse); human mPGES-1 hnRNA primers were designed to a portion of intron 2 and the adjoining exon 3, allowing for the detection of newly synthesized hnRNA. Each real-time PCR was conducted in triplicate using the Applied Biosystems 7000 Sequence Detection System with the following parameters: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. At completion, the melting curves were acquired by a stepwise increase in temperature from 55°C to 95°C to ensure that a single product was amplified in each reaction. Cyclophilin A levels were also measured concurrently as the internal control utilizing the following primers: human, 5′-CATCTCAAAAGCATACGGGTTC-3′ (forward) and 5′-GTCTGTCTTTGGCATTCTCG-3′ (reverse); mouse, 5′-GCCAGGTCTTACGAG-3′ (forward) and 5′-GCACCTGCTCAGAGG-3′ (reverse); rat, 5′-GCCACGATGAGGACCA-3′ (forward) and 5′-GGCTGAGCAGGTC-3′ (reverse). For real-time RT–PCR, the ΔΔCt method [42] was used for calculations in which the crossing point (Ct) values generates a ΔCt value. The difference between Ct values generates a ΔCt value. The difference between the ΔCt value for any given sample and the control sample generates the ΔΔCt value. 2−ΔΔCt provides the relative fold change for each sample compared with the control, which is normalized to 1. These values are then plotted as the relative fold induction.

Immunoprecipitation

HFL-1 cells were grown as described above and exposed to IL-1β at 2 ng/ml for 72 h. Total cell extracts were prepared in TNE lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 10 μg of aprotinin, 10 μg of leupeptin, 10 μg of pepstatin, 1 mM PMSF and 25 μM diithiothreitol) and incubated at 4°C overnight with an antibody against the indicated protein. Protein A/G–agarose beads (Santa Cruz Biotechnology) were washed four times in TNE lysis buffer, then incubated with the lysates at 4°C for 2 h. Bead complexes were washed four times with TNE lysis buffer, and proteins were eluted using 1× Laemmli buffer followed by immunoblot analysis with the indicated antibody.

Immunoblot analysis

Immunoprecipitates or 20 μg of total protein were separated on a 15% Tris/HCl polyacrylamide gel (Bio-Rad Laboratories)
and electrotransferred on to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked overnight at 4 °C with 5% (w/v) non-fat dried milk powder in TBST (Tris-buffered saline with Tween 20: 10 mM Tris/HCl, pH 7.5, 0.1% Tween 20 and 200 mM NaCl). A polyclonal antibody against mPGES-1 was added to the membrane, incubated at 4 °C overnight, washed three times with TBST, incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h, washed again three times with TBST, and subjected to ECL (enhanced chemiluminescence) (GE Healthcare).

**HS (DNase I-hypersensitive site) analysis**

HFL-1 cells were incubated in the presence or absence of 2 ng/ml IL-1β for 8 h, rinsed with PBS then trypsinized for 10 min at 37 °C. The cells were resuspended in 4 ml of permeabilization buffer (150 mM sucrose, 80 mM KCl, 35 mM Hapes, pH 7.4, 5 mM K2HPO4, 5 mM MgCl2, and 0.5 mM CaCl2) containing 0.1% L-α-lyso-lecinthin on ice for ~2.5 min. The reaction was stopped by the addition of 40 ml of permeabilization buffer and DNase I for 4 min at 37 °C. The cell suspension was digested with increasing concentrations of DNase I for 4 min at 37 °C. The reactions were terminated and genomic DNA isolated by the addition of DNA lysis buffer (4% SDS, 0.2 M EDTA and 800 μg/ml proteinase K), incubated at 50 °C for 3 h, followed by organic extractions, precipitation and resuspension in TE (10 mM Tris/HCl, pH 8.0, and 1 mM EDTA). The DNase I-digested samples were then digested with HindIII and size-fractionated on a 0.8% HGT agarose gel in TAE buffer, pH 7.8 (40 mM Tris, 3 mM sodium acetate, 1 mM EDTA and 4 mM NaOH) overnight at 40 V. The gel was then alkaline-denatured, electrotransferred on to a nylon membrane and cross-linked with UV light. The membrane was hybridized at 61 °C with an end-specific single-copy DNA probe, generated by PCR from the human mPGES-1 genomic clone, and radiolabelled using a random-primer DNA-labelling system. Hybridization and autoradiography were performed as described above.

**Plasmid construction and site-directed mutagenesis**

The mPGES-1 promoter constructs were generated by subcloning a human mPGES-1 promoter fragment, from 1086 bp or 416 bp upstream to 180 bp downstream of the mPGES-1 transcription start site, −1086/+180 (−1.1 kb) or −416/+180, into the HindIII and BamHI sites of the promoterless pUC12-based hGH reporter vector. The position of the transcription initiation site (+1) was based on updated cDNA sequence from NCBI Reference Sequence NM_004878.4 and thus the promoter numbering and positions of specific binding sequences differ from previous studies [34–39]. The following fragments were then subcloned upstream of the −1.1 kb mPGES-1 promoter at a newly generated MluI site labelled according to the distance from the mPGES-1 transcriptional start site in kb: −10.7/−6.4, −10.7/−9.6, −10.1/−9.0, −9.5/−8.5, −8.6/−6.4, −7.6/−6.4, −8.6/−8.1 and −8.1/−7.6. The −10.7/−6.4 fragment was also cloned into the −416/+180 promoter construct as well as the Ndel site of the hGH reporter vector containing the heterologous viral TK (thymidine kinase) promoter. The three potential C/EBPβ consensus sites in the −8.6/−8.1 fragment were deleted (Site 1, −8557/−8547; Site 2, −8399/−8388; Site 3, −8374/−8365) by QuikChange® site-directed mutagenesis (Stratagene). The deletion in the −1.1 kb promoter of the potential C/EBPβ site (−950/−933) or the two GC boxes for Egr-1 (−60/−42) were similarly deleted.

**Transient transfection**

Cells [(1−5)×104] were seeded on a 10-cm-diameter dish before transfection or treatment with cytokines. A 5 μg amount of the indicated plasmid was transfected into HFL-1 cells using FuGENE® 6 following the manufacturer’s instructions. To ensure equal transfection efficiency for each transfected construct cells were batch transfected, wherein 24 h post-transfection, each 10-cm-diameter plate of cells was split into two 10-cm-diameter plates and incubated overnight. At 40 h post-transfection, cells were not treated or stimulated with 2 ng/ml of IL-1β for 8 h.

**ChIP (chromatin immunoprecipitation) analysis**

ChIP was performed according to a modified protocol from Upstate Biotechnology using real-time RT-PCR for analysis of all samples as described previously [43]. The primers 5′-AGAGGAAATGGGAATGACTG-3′ (forward) and 5′-TCTTGTAGACCCAGACGTTG-3′ (reverse) were used to amplify the region from −8.6 to −8.1 kb (enhancer region); the primers 5′-CGGCAACTGTTGCTTTCTC-3′ (forward) and 5′-TTTGCAGCTCAGCAGCAAAGTA-3′ (reverse) were used to amplify the promoter region of human mPGES-1. The primers 5′-GCATCAAAAAACATCTCCCTCT-3′ (forward) and 5′-ACTCCAGTTGGCACCAGA-3′ (reverse) were used to amplify the 3′-UTR (untranslated region) and the primers 5′-AGAAGCCTAACATCACTCTCCT-3′ (forward) and 5′-ACAGCTCCTACAGCATAACCAG-3′ (reverse) were used to amplify the 5′-UTR of mPGES-1 as negative controls. All results are expressed as a fraction of the total isolated chromosomal DNA (input) before immunoprecipitation normalized to the anti-IgG control antibody.

**siRNA analysis**

HFL-1 or L2 cells were transfected with a final concentration of 100 nM SMARTpool® C/EBPβ siRNA (Dharmacon) using DharmaFECT™ 1 siRNA transfection reagent (Dharmacon) as described previously [44]. All SMARTpool® siRNAs are directed against four independent target sequences within each designated mRNA. Treatment with DharmaFECT™ 1 without siRNA was used to control for transfection-reagent-specific effects and a cyclophilin-specific siRNA (Dharmacon) was employed as a control for off-target effects of siRNA.

**Data analysis**

For all histograms, ‘relative fold induction’ is defined as the fold change of each sample as compared with the sample used for normalization. For statistical analyses, a one-tailed Student’s t test was used to determine inductions, whereas a two-tailed Student’s t test was used to determine differences in fold inductions. Student’s t tests were paired when comparing batch-transfected cells within each independent experiment and unpaired for all other analyses. Statistical significance is denoted when \( P < 0.05 \).

**RESULTS**

**Cytokine regulation of mPGES-1 in human lung fibroblasts**

To determine the effective concentration of IL-1β required to induce mPGES-1 gene expression in the human lung fibroblast cell line (HFL-1), cells were exposed to increasing concentrations
of IL-1β (0.5–10 ng/ml) for 8 h; total RNA was isolated and analysed by real-time RT–PCR for mPGES-1 mRNA expression (Figure 1A, left-hand panel). A 2 ng/ml concentration of IL-1β elicited the largest increase in mPGES-1 expression, approximately 8-fold, with this concentration being used for all subsequent experiments.

To demonstrate a corresponding increase in mPGES-1 protein levels, mPGES-1 was immunoprecipitated from control and IL-1β-stimulated HFL-1 cells followed by immunoblot analysis (Figure 1A, right-hand panel). Immunoprecipitation was utilized because our efforts to use two commercially available antibodies did not provide confident detection of mPGES-1 protein levels. The correct protein size was confirmed by overexpression of mPGES-1 following transfection with an mPGES-1 mammalian expression vector (results not shown).

A temporal analysis of IL-1β-dependent induction of mPGES-1 mRNA in HFL-1 cells revealed a maximal induction at 8 h (Figure 1B, top panels). Given that PGH₂, the product of the inducible COX-2 or PGH synthase 2, serves as the substrate for mPGES-1 production of PGE₂ [45], we also evaluated the induction of COX-2 in our cells in response to IL-1β (Figure 1B, bottom panel). As expected from the literature [45], COX-2 levels were significantly induced beginning 30 min after stimulation, thus identifying a potential link between the regulation of these two enzymes. To determine whether cytokine-dependent induction was due to an increase in de novo transcription, we quantitatively measured hnRNA levels by real-time RT–PCR as described previously [44] (Figure 1C). mPGES-1 hnRNA levels were increased ~6-fold within 30 min and reached a maximal induction of ~11-fold 1 h after IL-1β stimulation. These results demonstrate that the increase in mPGES-1 mRNA levels in response to IL-1β treatment is dependent on de novo mRNA synthesis.

**HS analysis of the human mPGES-1 chromatin structure**

Previous studies have demonstrated that the minimal mPGES-1 promoter confers a ~2–3-fold induction following treatment with a pro-inflammatory mediator [19,37,46]. We hypothesized that the regulation of mPGES-1 gene expression by IL-1β requires regulatory sequences in addition to the proximal promoter fragments due to the ~8-fold endogenous mRNA induction by IL-1β (Figure 1A), consistent with previous studies. To determine if there were, in fact, other regulatory sequences involved, we utilized HS analysis to identify altered regions of chromatin structure often associated with regulatory sequences. The right-hand side of Figure 2 illustrates such an analysis for a 13.3 kb HindIII fragment from the mPGES-1 locus using an adjacent 13.2 kb HindIII fragment from a region near the 5′ end (−6.4 kb) of the restriction fragment displays a constitutive HS (HS1) which maps to ~300 bp upstream of the transcriptional start site.

We next examined a different region of the mPGES-1 gene locus using an adjacent 13.2 kb HindIII fragment from −19.6 kb to −6.4 kb. This analysis detected an additional constitutive HS (HS2) ~11 kb from the probe which maps to a region near −8.6 kb (Figure 2, left-hand side). Our previous experience [47,48] has demonstrated that pre-existing constitutive HSs often harbour inducible regulatory factors.

**HS2 contains elements that co-operate with the basal mPGES-1 promoter and display stimulus-dependent enhancer activity**

To assess whether HS2 harbours functional regulatory activity, we placed a fragment extending from −10.7 to −6.4 kb in front of a −1.1 kb or −416 bp mPGES-1 promoter fragment coupled to an hGH reporter plasmid (Figure 3A). Both promoter constructs contain a well-studied Egr-1 site associated with basal and stimulus-dependent activation [34–39], whereas the −1.1 kb promoter fragment also harbours additional putative regulatory sequences [35,37]. As shown by Northern blot analysis (Figure 3A), each WT promoter construct alone exhibits both basal and inducible activity (~2.5-fold), whereas the fragment containing HS2 coupled to
IL-1β-responsive enhancer mediates mPGES-1 gene expression

Figure 2 Identification of alterations in the chromatin structure by HS analysis

Upper panel: a schematic diagram of the 5′ region of the mPGES-1 gene, indicating the location of three HindIII restriction sites spanning from −19.6 to +6.9 kb, relative to the transcriptional initiation site (+1). The arrows show the location of the single-copy probes used for indirect end-labelling of the 5′-end of two genomic restriction fragments, 13.2 and 13.3 kb. Lower panels: Southern blot analysis of the 13.2 and 13.3 kb fragments. ‘M’ denotes the molecular-mass marker (sizes in kb) and ‘−’ denotes no DNase I treatment. The triangle denotes increasing concentrations of DNase I in control and IL-1β-treated cells. The arrow on the right of each autoradiogram denotes the fragment size containing each HS site. For the left-hand panel, the probe hybridizes with an ∼11 kb fragment which maps to ∼−8.6 kb (HS2), whereas a ∼6.1 kb fragment is displayed in the right-hand panel mapping to ∼−300 bp (HS1).

either mPGES-1 promoter confers a significant increase in each of these activities, more closely resembling the induced levels of the endogenous mPGES-1 mRNA.

To address the increase in basal promoter activity when HS2 is coupled to the −1.1 kb promoter, we created a series of HS2 deletions which were coupled to this promoter fragment (Figure 3B) and evaluated them by transient transfection. A comparison of basal expression levels with those using the promoter alone revealed two regions, −10.1/−9.0 and −8.1/−7.6, which caused a statistically significant increase in the basal expression of the mPGES-1 promoter. To test whether the −10.7 to −6.4 kb fragment may also contain stimulus-dependent enhancer activity, the −10.7/−6.4 fragment was subcloned into an hGH vector that harbours a minimal viral TK promoter. A representative Northern blot analysis demonstrates the presence of an IL-1β-dependent enhancer-like activity in this fragment (Figure 3C, left-hand panel, inset). Densitometry results from four independent Northern blot analyses demonstrate a ∼5-fold increase in cytokine-stimulated TK promoter expression (Figure 3C, left-hand panel). Another criterion for a stimulus-linked enhancer is the ability of the fragment to function in an orientation-independent manner. The right-hand panel of Figure 3(C) illustrates real-time RT–PCR data from cells transfected with the TK/hGH reporter construct containing the −10.7/−6.4 fragment in the reverse orientation. These results (∼6-fold induction) demonstrate clearly that this region of the distal mPGES-1 promoter satisfies the criteria for a stimulus-dependent enhancer.

To demarcate the region with enhancer activity within the −10.7/−6.4 fragment, each of the seven deletion fragments described in Figure 3(B) coupled to the −1.1 kb mPGES-1 promoter were batch-transfected into HFL-1 cells to ensure equal transfection efficiency between control and treated plates (Figure 3D). Total RNA was analysed for hGH expression by real-time RT–PCR. The results demonstrate that an IL-1β-responsive enhancer exists between −8.6 and −8.1 kb (Figure 3D) and that this 500 bp fragment coupled to the −1.1 kb mPGES-1 promoter confers an IL-1β induction (∼6.5-fold) that more closely resembles the response of endogenous mPGES-1 mRNA to IL-1β (Figure 1, ∼8-fold) but is clearly statistically larger than the promoter alone (∼3-fold, \( P \leq 0.01 \)).

Identification of the IL-1β-dependent regulatory sequences responsible for induction of mPGES-1

In silico examination of the −8.6 to −8.1 kb region using transcription factor software (TESS: http://www.ncbi.nlm.nih.gov/ cgi-bin/tess/tess) to identify putative DNA-binding sites revealed three potential C/EBPβ consensus-like elements (Figure 4A). The sequence of this region was also compared across species, by aligning the mouse, rat and human sequences anchored at −1 by ClustalW analysis (EMBL) with sites 2 and 3 displaying 60–70% identity (Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/4430561add.htm). The physiological contribution of each putative C/EBPβ element on both basal (Figure 4B) and cytokine (Figure 4C) induction was determined by deleting each site (1, 2 and 3) individually and in combination, followed by transfection of each mutant construct into HFL-1 cells. Real-time RT–PCR from three independent experiments demonstrated that: (i) Site 1 has no functional role with regard to IL-1β induction (Figures 4B and 4C), (ii) Site 3 may be involved in basal expression (Figure 4B), and (iii) Site 2 is critically important in the IL-1β induction of mPGES-1 (Figure 4C). Most convincingly, whether Site 2 was deleted alone or in combination (Sites 1 and 2 or Sites 2 and 3), the fold induction by IL-1β returned to that of the promoter alone. The results in Figure 4(C) also support a potential role for Site 3 as a competitive binding site, since the deletion of Site 3 alone causes a statistically significant increase in IL-1β induction (∼10.5-fold) as compared with the WT enhancer/promoter construct (∼6-fold).

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Figure 3  HS2 contains elements that co-operate with the basal mPGES-1 promoter and display stimulus-dependent enhancer activity

(A) Top: the schematic diagram depicts the upstream 5' region of the mPGES-1 locus showing a −10.7/+6.4 kb fragment, used for subsequent cloning, containing HS2, as well as a −1.1 kb or −416 bp promoter fragment extending to +180 bp. Bottom: the −1.1 kb or −416 bp mPGES-1 promoter with or without the −10.7/+6.4 kb fragment was batch-transfected into HFL-1 cells before stimulation with 2 ng/ml IL-1β for 8 h. The membrane from Northern blot analysis was successively probed for the hGH reporter, mPGES-1 and the L7a loading control. (B) A series of overlapping fragments surrounding HS2 (−10.7/+6.4 kb, −10.1/+6.4 kb, −9.5/+6.4 kb, −9.5/−6.4 kb, −8.6/+6.4 kb, −8.6/−6.4 kb, −8.6/−8.1 kb, −8.6/−7.6 kb, −8.6/−6.4 kb) were subcloned 5' to the −1.1 kb mPGES-1 promoter coupled to hGH reporter gene, and batch-transfected into HFL-1 cells. Total RNA was subjected to real-time RT–PCR to detect hGH normalized to cyclophilin A mRNA. Relative fold induction is calculated from the mean ± S.E.M. of 3−4 independent experiments. (C) The constructs utilized in (B) surrounding HS2 were batch-transfected into HFL-1 cells, followed by treatment with IL-1β. Total RNA was subjected to real-time RT–PCR to detect hGH normalized to cyclophilin A mRNA. In order to compare respective constructs based on IL-1β responsiveness, the hGH levels of all untreated cells for each construct were set to 1. Relative fold induction is calculated from the mean ± S.E.M. of 3−4 independent experiments as described in the Experimental section and normalized to the respective untreated controls. **P ≤ 0.01 compared with the promoter alone. (D) The constructs utilized in (B) surrounding HS2 were batch-transfected into HFL-1 cells, followed by treatment with IL-1β. Total RNA was subjected to real-time RT–PCR to detect hGH normalized to cyclophilin A mRNA. In order to compare respective constructs based on IL-1β responsiveness, the hGH levels of all untreated cells for each construct were set to 1. Relative fold induction is calculated from the mean ± S.E.M. of 3−4 independent experiments as described in the Experimental section and normalized to the respective untreated controls. **P ≤ 0.01 compared with respective untreated samples. **P ≤ 0.01 compared with respective untreated samples. **P ≤ 0.01 compared with respective untreated samples.

The importance of Egr-1 in stimulus-dependent expression of mPGES-1 and its interaction with C/EBPβ

Naraba et al. [37] originally described the importance of the immediate-early gene transcription factor Egr-1 in the regulation of the human mPGES-1 gene by PMA through the interaction of Egr-1 with two GC boxes in the proximal promoter. Subbaramaiah et al. [38] confirmed the importance of these GC boxes and Egr-1 in the function of the mPGES-1 promoter in the human colon cancer cell line HCA7 in response to TNFα (tumour necrosis factor α). To address the functional importance of the Egr-1 sites in our cells, we analysed a construct where the two GC boxes from −416 bp to −60 bp were deleted (SGS1) in the human −1.1 kb mPGES-1 promoter construct compared with the WT promoter (Figure 5A). Deletion of the Egr-1-binding sites reduced the basal level of expression with induced levels still detectable. From computer analysis of this region, we also identified a putative C/EBPβ site at −0.9 kb in the human promoter with a similarly positioned site identified previously in the mouse promoter [37]. However, deletion of this site had no effect (Figure 5A). A double mutant, ΔEgr-1/ΔC/EBPβ, displayed results identical with those for the ΔEgr-1 mutant alone (Figure 5A).

We then evaluated the consequences of the Egr-1-binding site deletion alone or in the context of the C/EBPβ deletions with respect to basal expression (Figure 5B) and IL-1β induction using real-time RT–PCR (Figure 5C). Deletion of the Egr-1 site caused a 50% reduction in basal promoter activity (Figure 5B, upper panel). In addition, deletion of Site 1 in the context of the Egr-1 site deletion results in a further reduction (90%) in basal promoter activity. Similarly, deletion of both Site 3 and the Egr-1 site causes a 60% reduction in basal expression.

Deletion of Egr-1 sites in the promoter alone leads to a small (∼27%), but statistically significant, decrease in IL-1β induction consistent with the low level seen by Northern blot analysis in
IL-1β-responsive enhancer mediates mPGES-1 gene expression

A

-8630 AGCGAGACG GCGGCACTAC CAGCTGCTC CAGATGCTC CTTGACATT
-8560 AGCGAGACG TCAAGCTCC TTCGCTCAG CAGATGCTC CCAGACACG
-8530 CTTCGGGCCC ACACCACTAT GAGCAATTC AAGTAAACA CTAAACAGAC
-8480 AGACATGCY TTCTGATTT CTTTCTACAA ATACCTACG AACTCCGT
Site 1
-8430 GTCCAGATCC AGCCATGTAC TATAGAAGCT ATATTCTGGA ACGACACAC
-8360 AGACATGCY CATCAGTTCC TCCACCCCG CGCCACCGC CACGACACAC
-8330 ATGAGAAGCT CAGGCCCC ATCCGCCC CGCCAGC CGCAGACACAC
-8260 AATATGTGCT TCTGCTGTTA TGCTGCTGAA CATCTGACG ATTATGATG
Site 2
-8230 CTTGAGATTCA TCAATAAACA TTCAATAGCA CCCTAGCTCC TTCCACCTAC
-8180 CAGAAGCGCT AGGGAGAA AGCCCTTGA CAGACAGAAT GAAATACAG
-8130 GTGTAAGGGAG ATGGGACCTG TCAGCTCTAG CTTGAGACG ACCTCGCTCT

B

-8.6
-8.1
-1 kb Promoter

- Site 1
- Site 2
- Site 3

0 1 2 3 4 5
Relative Fold Induction

C

-8.6
-8.1
-1 kb Promoter

- Control
- IL-1β

0 1 2 3 4 5
Relative Fold Induction

Figure 4 Identification of the IL-1β-dependent regulatory sequences responsible for induction of mPGES-1

(A) The sequence of the —8.6/−8.1 fragment is shown with the location of three potential C/EBPβ consensus binding sites (Site 1, −8557 to −8547; Site 2, −8399 to −8386; Site 3, −8374 to −8365) in bold. Identified using TESS software based on the TRANSFAC database [59]. The TRANSFAC database utilizes the following C/EBPβ consensus sequence for the analysis: G(A/T)(A/C/G)(T/G)(N/C/G/T)(G/C/T)(C/T)AA G(T/G) NNA(C/T). The underlined regions (Sites 1−3) were deleted within the —8.6/−8.1 fragment coupled to the —1 kb mPGES-1 proximal promoter. (B) Basal expression with single and double deletions of Sites 1−3 was compared with the WT fragment by transient transfection in HFL-1 cells and analyzed by real-time RT–PCR. WT C/EBPβ sites are represented as diamonds (●) and an X indicates a deleted site. Relative fold induction is calculated from the mean ± S.E.M. 2−ΔΔCt values (3 ≤ n ≤ 14) as described in the Experimental section and normalized to the WT enhancer construct. (C) IL-1β-dependent induction was evaluated in the promoter alone and in the context of single and double deletions of Sites 1−3 in the enhancer by transient transfection in HFL-1 cells and analyzed by real-time RT–PCR. WT C/EBPβ sites are represented as diamonds (●) and an X indicates a deleted site. Relative fold induction is calculated from the mean ± S.E.M. 2−ΔΔCt values (3 ≤ n ≤ 13) as described in the Experimental section and normalized to the respective untreated samples. *P ≤ 0.05, **P ≤ 0.01 compared with respective untreated samples. †P ≤ 0.05, ††P ≤ 0.01 compared with the fold induction of the WT —8.6/−8.1 fragment coupled to the promoter.

C/EBPβ is required for the IL-1β-dependent induction of mPGES-1 mRNA

To establish a functional role for C/EBPβ in the IL-1β-dependent induction of mPGES-1, we evaluated the response in WT MEFs and MEFs derived from C/EBPβ−/− mice using real-time RT–PCR [49] (Figure 7A). IL-1β treatment caused a 2−fold induction of mPGES-1 mRNA levels in WT MEFs, with no induction observed in the C/EBPβ−/− MEFs.

To verify further the functional importance of C/EBPβ, we employed species-specific C/EBPβ siRNA pool to knock down endogenous C/EBPβ mRNA and protein levels in both a rat pulmonary epithelial cell line (L2) (Figure 7B) and human HFL-1 cells (Figure 7C). It should be noted that previous studies demonstrate that Egr-1 siRNA only partially inhibits cytokine induction of mPGES-1 [39]. C/EBPβ knockdown in L2 cells causes the reduction of the IL-1β-dependent induction (Figure 7B) to a level comparable with that observed for the Egr-1-linked proximal promoter fragment (Figures 3A, 3C and...
Figure 5  The importance of Egr-1 in stimulus-dependent expression of mPGES-1 and its interaction with C/EBPβ

(A) Top: Schematic diagram of the −1.1 kb mPGES-1 promoter fragment illustrating the documented Egr-1 and putative C/EBPβ transcription factor-binding sites. Bottom: binding sites were deleted individually (ΔEgr-1 (−60/−42) and ΔC/EBPβ (−950/−933)) or in combination (ΔEgr-1/ΔC/EBPβ) from the −1.1 kb promoter fragment, and each mutant construct was batch-transfected into HFL-1 cells, followed by Northern blot analysis for hGH, mPGES-1 and the L7a loading control. (B) Top: basal expression was evaluated in the context of the WT Egr-1 (Egr-1) promoter-binding site compared with an Egr-1 deletion (Δ1Egr-1). *P ≤ 0.05 compared with WT promoter. Bottom: basal expression for the enhancer coupled to the Egr-1 promoter deletion was compared with constructs harbouring WT (●) or single deletions in C/EBPβ Sites 1–3 (X). Each construct was transfected into HFL-1 cells, and total RNA was evaluated by real-time RT–PCR. Relative fold induction is calculated from the mean ± S.E.M. 2^{−ΔΔCT} values (3 ≤ n ≤ 14) as described in the Experimental section and normalized to the respective untreated samples. *P ≤ 0.05, **P ≤ 0.01 compared with respective untreated samples. †P ≤ 0.05 compared with the fold induction of the WT promoter alone. (C) WT binding sites for Egr-1 (Egr-1) in the promoter or C/EBPβ (C/EBPβ) in the enhancer were selectively deleted (Δ). Each construct was transfected into HFL-1 cells, with or without IL-1β, and total RNA was evaluated by real-time RT–PCR. Relative fold induction is calculated from the mean ± S.E.M. 2^{−ΔΔCT} values (3 ≤ n ≤ 14) as described in the Experimental section and normalized to the respective untreated samples. *P ≤ 0.05 compared with the fold induction of the WT promoter alone.

Figure 6  Association of Egr-1, C/EBPβ and RNA polymerase II with the mPGES-1 gene by ChIP

(A) HFL-1 cells were stimulated with 2 ng/ml IL-1β for 8 h, and subjected to ChIP as described in the Experimental section with control IgG-, RNA polymerase II (Pol II)- or Egr-1-specific antibodies. Pol II or Egr-1 were analysed at both the promoter and enhancer regions using real-time PCR. Relative fold induction is calculated from the mean ± S.E.M. 2^{−ΔΔCT} values (n = 2 for Egr-1 treated with IL-1β, n = 3 for all other samples) as described in the Experimental section and normalized to IgG. *P ≤ 0.05, compared with untreated samples. (B) HFL-1 cells were stimulated with 2 ng/ml IL-1β for 0, 4 or 8 h and subjected to ChIP with control IgG- or C/EBPβ-specific antibodies at the enhancer region or a control region in the 3′-UTR. Inset: ChIP analysis of C/EBPβ at the promoter. Relative fold induction is calculated from the mean ± S.E.M. 2^{−ΔΔCT} values (n = 3) as described in the Experimental section and normalized to IgG. *P ≤ 0.05, compared with untreated samples.

5A). Verification of endogenous C/EBPβ protein knockdown in L2 cells using this siRNA pool has been published previously [43]. The top panels of Figure 7C illustrates a representative immunoblot analysis of HFL-1 cells showing knockdown of C/EBPβ protein. Analogous to the rat epithelial cells, knockdown of C/EBPβ in human fibroblasts exhibited a very similar reduction in the IL-1β response (Figure 7C). As a negative control, a human cyclophilin B siRNA pool was also evaluated by immunoblot analysis with no significant knockdown of C/EBPβ (results not shown).

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DISCUSSION

The enhanced risk of cardiovascular events, stroke and myocardial infarction associated with selective COX-2 inhibitors has been linked to the redirection of prostacyclin and thromboxane synthesis [50]. These adverse consequences have led to increased interest in the terminal synthases, especially the regulated role of mPGES-1 in the synthesis of the central prostaglandin, PGE2. Therefore mPGES-1 is a promising target for next-generation anti-inflammatory drugs. In mild asthmatics, PGE2 can attenuate allergen-induced airway responses, hyper-responsiveness and inflammation [51]. Lundequist et al. [52] have also demonstrated the protective effects of mPGES-1 and its product, PGE2, by reducing pulmonary vasculature remodelling during allergen-induced pulmonary inflammation. Our efforts have therefore focused on obtaining a detailed understanding of the mechanisms controlling physiological levels of mPGES-1 which we predict will afford the opportunity to manipulate these levels at the point of transcriptional regulation.

The literature on mPGES-1 gene regulation implies that proximal promoter elements, namely two Egr-1-associated GC boxes, are adequate and sufficient to mediate the induction by PMA and pro-inflammatory cytokines [26,37–39,53,54]. We found that these results do not adequately explain the total increase in mRNA observed following cytokine treatment. Although a proximal promoter construct can confer a ~2–3-fold induction following cytokine treatment (Figures 3–5), our steady-state analysis of mPGES-1 mRNA levels revealed that IL-1β causes a significantly larger increase of ~9-fold (Figures 1A and 1B), consistent with that of other laboratories [26].

To identify additional regulatory regions, we utilized HS analysis which led to the identification of an HS at ~8–9 kb 5′ (Figure 2). Functional deletion pointed to a region (~8.6/8.1) with IL-1β-dependent enhancer activity (Figures 3B and 3C). Of most relevance, this region in conjunction with the proximal promoter more closely recapitulates the endogenous mPGES-1 induction levels. Characterization of this element implicated a specific C/EBPβ site (Site 2) that, when deleted, significantly reduced the induction to levels consistent with the promoter alone (Figure 4). ChIP data verified further the constitutive association of Egr-1 with the proximal promoter, inducible association of RNA polymerase II to the promoter, as well as stimulus- and time-dependent association of C/EBPβ to the distal enhancer. Both knockdown and knockdown studies demonstrated the critical functional importance of C/EBPβ to mPGES-1 gene regulation (Figure 7). To our knowledge, the only previous association between C/EBPβ and mPGES-1 was indirect studies in C/EBPβ-deficient mice. In one study involving a microarray analysis and Northern blot analysis in LPS/IFNγ (interferon γ)-stimulated peritoneal macrophages from WT mice, C/EBPβ-deficient mice and C/Ebpβ<sup>M1A/M1a</sup> mice, mPGES-1 regulation was indirectly linked to the 34 kDa isof orm of C/EBPβ [55]. Uematsu et al. [56] also showed that induction of mPGES mRNA and production of PGE<sub>2</sub> in response to LPS in C/EBPβ-deficient macrophages was...
abolished. We therefore feel that the combination of our deletion analysis, ChIP results and knockdown studies with C/EBPβ convincingly demonstrate a direct relevance of this member of the C/EBP family through a novel distal enhancer in mPGES-1 regulation.

Our results confirm the importance of Egr-1 to mPGES-1 promoter activity and, most importantly, highlight the requirement of a distal C/EBPβ-dependent enhancer to recapitulate the IL-1β-induction of the endogenous gene. To date, Damm et al. [57] and Zhang et al. [58] provide the only implication that C/EBPβ and Egr-1 can interact on the basis mostly of in vitro studies. Our results substantiate directly the interaction based on co-immunoprecipitation from endogenously expressed protein in intact cells (Figure 5C). Zhang et al. [58], however, do demonstrate that Egr-1 can only co-operate with C/EBPβ and not other members of the C/EBP family [58] which is consistent with our studies in C/EBPβ−/− MEFs along with siRNA knockdown results (Figure 7).

Figure 7(D) depicts a summary of the 5′ promoter region of the mPGES-1 gene. We have identified two distal elements (−10.1 to −9.0 and −8.1 to −7.6) that increase basal expression when coupled to the promoter as well as Sites 1 and 3 in the enhancer that, when deleted in conjunction with deletion of Egr-1 in the promoter, dramatically decrease basal expression. This summary also defines the importance of C/EBPβ Site 2 along with the co-operation of Site 3 in the cytokine-dependent induction of mPGES-1. Taken together, the results of the present study define a novel distal enhancer region controlling IL-1β-induction of mPGES-1 with a unique role for C/EBPβ. Additionally, the present study supports the role of Egr-1 in mPGES-1 promoter regulation and codifies the association between Egr-1 and C/EBPβ. Most importantly, our results with the enhancer closely recapitulate the IL-1β-induction of endogenous mPGES-1 gene expression, thus providing a more complete understanding of the complex regulatory mechanism controlling this gene beyond the previously characterized promoter.

**AUTHOR CONTRIBUTION**

Jewell Walters performed the majority of both the experimental design and completion of each study. Justin Bickford participated in experiments, analysis and writing and editing of the paper before submission. Kimberly Newsom assisted with ChIP and siRNA studies. Dawn Beachy assisted with many of the experiments. Sarah Barilovits contributed to overall design and data interpretation. John-David Herlihy assisted with initial concept and design of experiments. Harry Nick oversaw all of the studies and drafted and approved the paper.

**ACKNOWLEDGEMENTS**

We thank the members of the Nick and Killberg laboratories for helpful discussions and Dr Sherri Y. Liang for assistance with Figures.

**FUNDING**

This work was supported by the National Institutes of Health [grant numbers R37HL067456 and RO1HL39593 (to H.S.N.)].

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SUPPLEMENTARY ONLINE DATA

Regulation of human microsomal prostaglandin E synthase-1 by IL-1β requires a distal enhancer element with a unique role for C/EBPβ

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Figure S1 Alignment of rat, mouse and human mPGES-1 enhancer regions with ClustalW (EMBL) with —1 as the 3′ anchor/ending site

Received 6 October 2011/19 January 2012; accepted 20 January 2012
Published as BJ Immediate Publication 20 January 2012, doi:10.1042/BJ20111801