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Human glutathione dependent prostaglandin E synthase: gene structure and regulation

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Abstract A P1 clone containing the gene for human glutathione dependent PGE synthase (PGES) was isolated and characterized. The gene is divided into three exons, spans 14.8 kb and was localized to chromosome 9q34.3. In A549 cells, the protein and activity levels of PGES were increased by interleukin-1β. This increase was prevented by phenobarbital. Reporter constructs containing the 5′-flanking region of exon 1, which exhibited strong promoter activity, responded accordingly, except that interleukin-1β induced a transient increase followed by a decrease. As cyclooxygenase 2 expression has been reported to respond in a similar fashion, a transcriptional regulatory basis for the observed co-regulation with PGES is implied. The strong down-regulation by phenobarbital raises important issues concerning its mechanisms of action.

Key words: Prostaglandin; PGE synthase; MAPEG; Gene; Chromosomal; Phenobarbital

1. Introduction

The isoenzymes cyclooxygenase (COX)-1 and COX-2 catalyze the double oxygenation and reduction of arachidonic acid, leading to the formation of prostaglandin endoperoxide H_2. This intermediate serves as substrate for several terminal prostanoid synthases like the recently characterized human glutathione dependent prostaglandin E synthase (PGES) [1]. This enzyme represents an inducible member of the MAPEG superfamily [2]. Other members are microsomal glutathione transferase 1 (MGST1), MGST2 and MGST3, which all are glutathione transferases and glutathione dependent peroxidases. The family also includes 5-lipoxygenase activating protein (FLAP) and leukotriene C_4 synthase, which are crucial for leukotriene biosynthesis. PGES is most closely related to MGST1, demonstrating 38% identity on the amino acid sequence level. The gene encoding MGST1 resides on chromosome 12 [3] and consists of three coding exons and one first non-coding exon [4]. Later studies have demonstrated the presence of several alternative non-coding first exons [5,6] of which one is predominant. Other MAPEG genes (FLAP and LTC_4 synthase) that have been characterized consist of five exons.

The chromosomal localization of all MAPEG members except PGES is known. LTC_4 synthase, FLAP, MGST2 and MGST3 map to chromosomes 5q35, 13q12, 4q28–31 and 1q23, respectively [7–10]. Interestingly, LTC_4 synthase, which plays a role in bronchial asthma, localizes in a region harboring several genes involved in inflammation [7]. Cyclooxygenases 1 and 2 have been localized to chromosomes 9q32–33.3 and 1q25.2–25.3 [11–13].

Cyclooxygenase 2 and PGES are induced by the proinflammatory cytokine interleukin-1β (IL-1β) suggesting the possibility of a common gene regulatory mechanism [1]. Several reports have demonstrated that PGES, COX-2 and phospholipase A_2 form an inducible pathway for efficient and preferred PGE_2 production [14,15].

Here we report the PGES gene structure[1], localization and certain regulatory properties. These data form an essential basis for obtaining a better understanding of the link between COX-2 and PGES in particular, and the interplay between MAPEG members, cyclooxygenases and lipoxygenases in inflammation and other pathophysiological conditions in general.

2. Materials and methods

2.1. Cell culture

A549 human alveolar cells were maintained in DMEM containing 4500 mg/l glucose, 10% fetal calf serum, 1 mM sodium pyruvate, 100 U penicillin/ml and 100 μg streptomycin/ml.

2.2. Genomic cloning and chromosomal localization

Genomic cloning and subsequent chromosomal localization was performed by Genome Systems, Inc. A P1 genomic library was screened by hybridization using a cDNA probe consisting of the coding region of PGES that was labeled by random priming. The P1 clone obtained was denoted: 18096 (96) with clone address: DMP-HFF#1-569G12. Purified DNA from this clone was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2×SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI for one color experiments. Probe detection for two color experiments was accomplished by incubating the slides

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Abbreviations: PGES, prostaglandin E synthase; PGD synthase, prostaglandin D synthase; MGST1, microsomal glutathione transferase 1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PB, phenobarbital; AHR, aryl hydrocarbon regulatory element; COX, cyclooxygenase; MAPEG, membrane associated proteins involved in eicosanoid and glutathione metabolism; IL-1β, interleukin-1β

[1] Sequence information is deposited with the following accessions numbers: AJ271802, AJ271803, AJ271804, exons 1–3, respectively. Approved gene symbol: PTGES. Name: prostaglandin E synthase. Alias: PGES, MGST-IV, PIG12, MGST1L1, MGST1-L1.

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in fluoresceinated antidigoxigenin antibodies as well as avidin Texas red followed by counterstaining with DAPI. The initial experiments resulted in the specific labeling of the long arm of a group C chromosome that was believed to be chromosome 9 on the basis of size, morphology, and banding pattern. A second experiment was conducted in which a biotin labeled probe, which is specific for the heterochromatic region of chromosome 9, was cohybridized with clone 18096. This experiment resulted in the specific labeling of the heterochromatin in red and the long arm in green of chromosome 9. Observation of specifically labeled chromosomes 9 demonstrated that clone 18096 is located at the terminus of the long arm of chromosome 9, an area that corresponds to band 9q34.3 [16]. A total of 80 metaphase cells were analyzed with 71 exhibiting specific labeling. Our chromosomal localization is in agreement with preliminary AC007936 from which primer sequences were constructed, but is in conflict with chromosomal localization assigned for preliminary AC015667.

2.3. Organization and sequence of the PGES gene

The sequence of the human PGES gene was determined by PCR and sequence analysis according to the strategy shown in Fig. 1A, using the P1 clone 18096 as template. All exons and exon/intron boundaries were sequenced in both directions. PCR reactions were performed in 1.5 mM MgCl, 0.15 mM dNTP, 10% DMSO, 0.5 U Taq Polymerase (Sigma) and were carried out in 30 cycles, each involving denaturation at 94°C, 45 s, annealing at 53°C, 45 s, elongation at 72°C, 1 min, followed by 7 min elongation. The PCR products were purified using high pure PCR purification kit (Boehringer, Germany) and the sequence analyzed using the big dye terminator kit (Perkin Elmer) and an ABI model 373 DNA sequencer.

2.4. Gene reporter constructs

Two human PGES promoter fragments including −651/−20 and −190/−20 (numbering the translation start site +1) were generated by PCR amplification, using a P1 clone as template, two sense primers (oligonucleotides U-promKpnI and U-promSacI, Table 1) and one common antisense primer (oligonucleotide L-promBglII, Table 1). The PCR products were cleaved with the appropriate restriction enzyme, gel-purified (Pharmacia) and subcloned into the *Kpn*I/*Bgl*II and *Sac*I/*Bgl*II sites of pGL2Basic reporter vector (Promega), resulting in the constructs pGL2–651 and pGL2–190 respectively. The sequences were verified by sequence analysis as described above. Plasmids were purified using the Qiagen endotoxin free kit.

2.5. Transfection assay

The A549 cells were plated in 35 mm 6-well plates at 4×10^5 cells per well and incubated overnight. The cells were transfected using Superfectin (Qiagen), 2.5 μg of PGES reporter construct and 5 μg of β-galactosidase control vector (Promega). Cells were incubated for 3 h at 37°C, then transfection solution was replaced with fresh media. 48 h later, cells were harvested and luciferase and β-galactosidase activities were determined. Transfected cells were treated with IL-1β, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and phenobarbital (PB) for 16 h before analysis. IL-1β was added in the presence of 2% FCS. Luciferase activity was determined using luciferase assay reagent (Promega) and β-galactosidase activity was measured using ortho-nitrophenyl β-D-galactopyranoside (Sigma) as substrate. All values were corrected for endogenous galactosidase activity. The luciferase value was always divided by the β-galactosidase value in order to correct for transfection efficiency.

2.6. Western blot and PGES activity analysis

The method for preparing microsomes, analyzing for PGES activity as well as protein expression by Western blot has been described in detail [1].

2.7. Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted with Trizol (Life Technology) from A549 cells incubated 0, 2, 4 and 6 h in the presence of IL-1β (1 ng/ml). RT reactions were performed using 5 μg of RNA, 1 μl of an poly(dT)-oligo (Stratagene) and 200 units of SuperscriptTMII (Life Technology). For amplification of PGES, exon 3 specific primers U-2MGST4 and L-2MGST4 (Table 1) were used. As a control, constitutively expressed human β-actin mRNA was also amplified with the primers β-sense and β-antisense (Table 1). PCR reactions were performed as described above. All PCR products were analyzed on 1% agarose gels.

2.8. Sequence analysis

Computer analysis of the 5′-flanking region of the human PGES for potential transcription factor binding sites was performed with MatInspector 2.2 [17].

3. Results

3.1. Genomic cloning and chromosomal localization

Using the coding sequence of PGES as probe, a genomic P1 library was screened and a genomic clone was obtained. This P1 clone (18096) was subsequently used to characterize the gene and promoter (see below) but also as a probe for chromosomal localization by fluorescence in situ hybridization. Based on cohybridization with another probe specific for the heterochromatic region of chromosome 9 and fractional length measurements, it was concluded that the PGES genomic clone is located at the terminus of the long arm of chromosome 9, an area that corresponds to band 9q34.3.

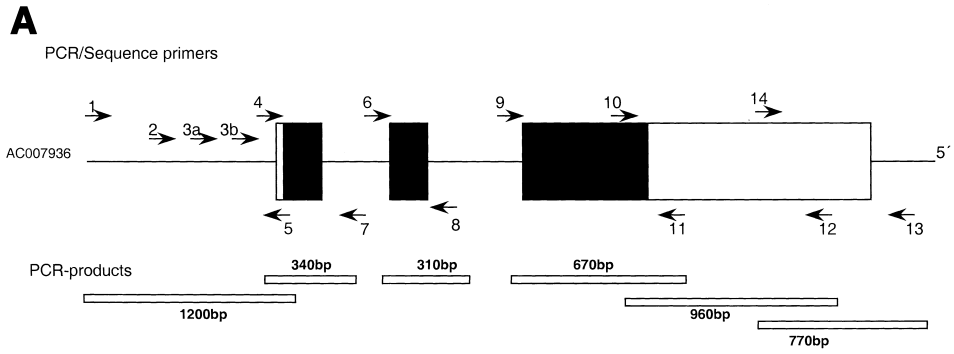
3.2. Gene organization

Fig. 1B contains the complete sequence of the human PGES exons, together with intron–exon junctions and 632 bp upstream of exon 1. As shown, the PGES gene is split into three exons (≈136, 83 and 1526 nt) by two introns and spans a region of approximately 14.8 kb. Exon–intron junctions follow the GT-AG rule except for the 5′-site of intron 2, which consists of GC instead of GT. The intron sizes are 4.2 kb and 8.8 kb respectively. A 682 bp fragment containing the 5′-flanking region of exon 1 is shown in Fig. 1B. This putative promoter is GC-rich and lacks a TATA box at a functional site. Computer analysis of the promoter revealed numerous potential transcription factor binding sites. Two GC-boxes, two tandem barbie boxes and an AHR (aryl hydrocarbon response element) were identified. The 3′-end of exon 3 con-

Table 1
Sequences of oligonucleotides

Primer	Fig. 1A	Sequence[a]
PCR/sequence analysis (Fig. 1)		
U-prompges1	1	ggctggaattacaggtgtga
U-promPGEseq	2	agaggcccctggtattccttg
U-PGES-1	4	gtgctggctgcaggaaggct
L-PGES-1	7	tgctcacctcccagccctgca
U-PGES-2	6	aactgcacctctgtcctgcca
L-PGES-2	8	ggaacacagaaaacctcagcc
U-PGES-3	9	gatttgagtgtgtgatatgcc
L-PGES-3	11	ttagctgaaggattttctatc
L-PGES-3b	12	gcaacatggtgaacccgtct
U-2MGST4	10	aacgacatggagaccatctac
U-PGES-3c	14	ggattacaggcgcgtatcac
L-PGES-3d	13	cagggaacaccaggaacgcg
Exon specific primers RT-PCR		
U-2MGST4		aacgacatggagaccatctac
L-2MGST4		acatcaagtccccaggtatagcc
β-Actin specific primers		
U-sense		gattcctatgtgggcgacgag
L-antisense		ccatctcttgctcgaagtcc
Reporter construct primers		
U-promKpn1	3a	gagaggtacccagataaact
U-promSac1	3b	gcttcttggagctccggcaa
L-promBgl2	5	tctctcagatctgtgggtgtg

[a]Restriction enzyme sites used for cloning are underlined.



B

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ACCCAGATAAACTAGGATAATTTCTCCCATCTCAAATCCTTACGAAATCCCATGTGAAAAGTCCCTTTTGCCACATAGTC
ACAGTCACGGGTTCTAGGGATTGGGACATGGATGTCTTTAGGGGTGGAGGGCGTCCTTCAGCCTGCCACAGATGGCAAAG
TCATGCCATTGTGGCTGCAGCTCCATTGTCCAGGCTGAGTGTGGGGGCGCATGGCGTGGTTCTCATGCCCACCACCTACC
CTGACCCTGCGGAGGTGGGCAGGAGGCAGCGATAATCACCACGTGCAGAGGTCCTGCCAGGTGCCCAGCCTCATCATTCC
CTAGAAGGCAGGCACCACCACATGCTCCCACACTGTAGATAAGGAAACAGCTTTGAAACAGATTTGAAAGTGTTTGAGGA
TTTGCCTGGAAGGAGGGCTCCAGAGCCAGGGACTGGGTCCGGGACACCCGGAGACTCTCTGCTTCTTGGAGCTCCGGCAA

                                                    GC-BOX      GC-BOX
CTGCTTGTCTTTCTCTTCACAGGAGAAGGGGGCCCTGCTGCCGCTGCCGTGGGGCGGGgcgtgGGGCGGTGCTGGCTGCAG
                                                          AHR
                  BARBIE-BOXES
GAAGGCTCTCCCCTCCCACTTTGTGCTTTCCCTGCAGGCTGCTCCTCTGTCGAGCTGATCACACCCACAGTTGAGCTGCG

CTGGCCAGAGATGCCTGCCCACAGCCTGGTGATGAGCAGCCCGGCCCTCCCGGCCTTCCTGCTCTGCAGCACGCTGCTGG
TCATCAAGATGTACGTGGTGGCCATCATCACGGGCCAAGTGAGGCTGCGGAAGAAGgtatgtgcctccctgggggtcccc
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                                    --4.2 kb--
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TGGGGAAGCTGCGGGCACCCATCCGCTCCGTGACCTACACCCTGGCCCAGCTCCCCTGCGCCTCCATGGCTCTGCAGATC
CTCTGGGAAGCGGCCCGCCACCTGTGACCAGCAGCTGATGCCTCCTTGGCCACCAGACCATGGGCCAAGAGCCGCCGTGG
CTATACCTGGGGACTTGATGTTCCTTCCAGATTGTGGTGGGCCCTGAGTCCTGGTTTCCTGGCAGCCTGCTGCGCGTGTG
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CTGGCCATCTGTATTTTTTTTTTTTCCAAGACAGAGTCTTGCTCTGTTGCCCAAGCTGGAGTGAAGTGGTACAATCTGGCT
CACTGCAGCCTCCGCCTCCCGGGTTCAAGCGATTCTCCCGCCTCAGCCTCCTGAGTAGCTGGGATTACAGGCGCGTATCA
CCATACCCAGCTAATTTTTGTATTTTTAGTAGAGACGGGTTCACCATGTTGCCCAGGAGGGTCTCGAACTCCTGGCCTCA
AGTGATCCACCGGCCTCGGCCTCCCAAAGTGCTGGGATGACAGGCATGAATCACTGTGCTCAGCCACCATCTGGAGTTTT
AAAAGGCTCCCATGTGAGTCCCTGTGATGGCCAGGCCAGGGGACCCCTGCCAGTTCTCTGTGGAAGCAAGGCTGGGGTCT
TGGGTTCCTGTATGGTGGAAGCTGGGTGAGCCAAGGACAGGGCTGGCTCCTCTGCCCCCGCTGACGCTTCCCTTGCCGTT
GGCTTTGGATGTCTTTGCTGCAGTCTTCTCTCTGGCTCAGGTGTGGGTGGGAGGGGCCCACAGGAAGCTCAGCCTTCTCC
TCCCAAGGTTTGAGTCCCTCCAAAGGGCAGTGGGTGGAGGACCGGGAGCTTTGGGTGACCAGCCACTCAAAGGAACTTTC
TGGTCCCTTCAGTATCTTCAAGGTTTGGAAACTGCAAATGTCCCCTTGATGGGGAATCCGTGTGTGTGTGTGTGTGTG
TGTGTGTGTGTGTGTGTGTTTTCTCCTAGACCCGTGACCTGAGATGTGTGATTTTTAGTCATTAAATGGAAGTGTC
TGCCAGCTGGGCCCAGCACCTCGtcttgggttgcgcgttcctggtgttccctgctgcccaagctggcctgatgctc
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Fig. 1. Structure and sequence of the human PGES gene. A: Structure of the human PGES gene. Introns and flanking sequences are represented by solid lines. Exons are represented by boxes; the open boxes and solid boxes indicate the non-coding regions and coding regions, respectively. Beginning of first exon is unknown. Also indicated are the primers used in PCR analysis, and the PCR products used for sequence analysis. B: The nucleotide sequence of the human PGES gene, exons (uppercase bold letters) and partial introns (lowercase letters) and 632 bp of the 5′-flanking region (uppercase). GC-boxes and barbie boxes are underlined and an AHR site is indicated with italics. The predicted polyadenylation signal and ATG translation start site are underlined.

tains a polyadenylation signal (underlined in Fig. 1B) 32 nt upstream of the polyA observed in cDNA clone AF027740.

Our sequence is in general agreement with a preliminary deposited sequence (AC007936, contig nt: 100 506–117 518), from which intron sizes were deduced, with an exception in the 3′ non-coding end of the gene where a GT-dinucleotide repeat is located. In our P1 clone 22 repeats were determined whereas in clone AC007936 24 repeats were noted. Two nt in AC007936, A16 and A49 were determined as C in our P1 clone (numbered from the initiator ATG). A nucleotide difference in the coding region (T183C), as compared to cDNA clone AF027740 was also identified, which does not alter the amino acid sequence. Three nucleotides, AGG at positions +165–167 in the cDNA clone coding region AF010316, are not present in our P1 genomic sequence. Several differences in the 3′ region to AC007936 and AF027740 were also noted.

3.3. Transcriptional activity of the putative PGES gene promoter

Transcriptional activity of the 5′-flanking region of human

PGES gene was examined in A549 cells, transiently transfected with reporter constructs containing 190 bp and 651 bp of the putative promoter (pGL2–190 and pGL2–651) and a promoterless vector (pGL2Basic) as negative control.

As shown in Fig. 2A both the pGL2–190 and pGL2–651 constructs increase luciferase expression, by 25- and 35-fold, respectively compared to the negative control. It appears that the cis-acting elements needed for the basal expression are included in the 171 bp fragment. This region contained two potential GC-boxes 119 and 107 bases upstream from the translation start site. Since the 5′-flanking region contains putative cis-acting elements such as barbie boxes and an AHR site, we stimulated transfected A549 cells with chemicals known to act via these regulatory elements. TCDD did increase luciferase expression 1.5-fold whereas 0.2 mM and 2 mM phenobarbital decreased expression 0.7-fold and almost completely (Fig. 2B).

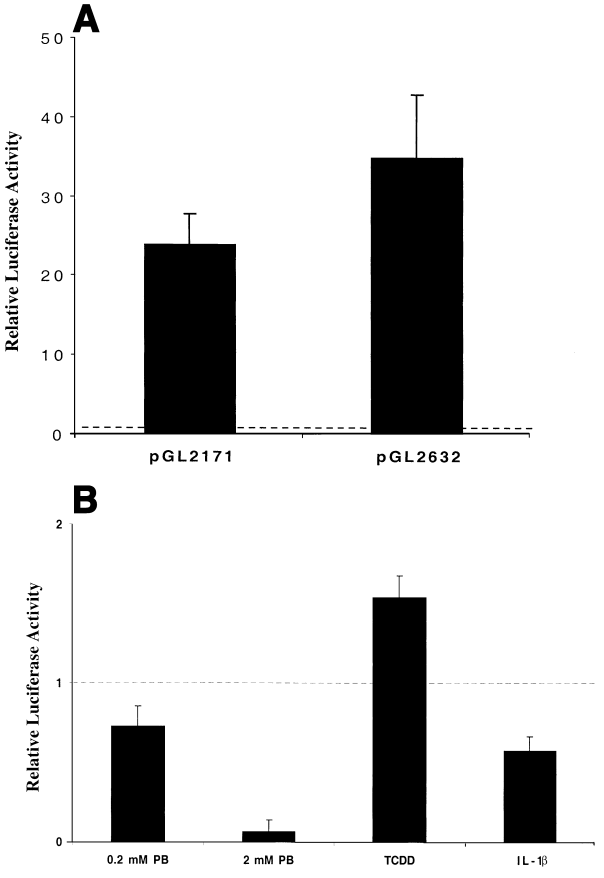

Fig. 2. Functional analysis of the human PGES promoter in A549 cells. A: Human PGES promoter fragments (−651/−20 and −190/−20) were subcloned upstream of the firefly luciferase reporter gene in the pGL2Basic vector. A549 cells were transiently transfected with the constructs. As a negative control the cells were transfected with promoterless plasmid pGL2Basic. The bar graph represents mean ± S.E.M. from three experiments performed in duplicate and the luciferase expression was normalized to the transfection efficiency by β-galactosidase expression. Data are expressed in relation to the promoterless plasmid. B: Effects of TCDD, phenobarbital and IL-1β on the transcriptional activity of the PGES 5′-flanking region. A549 cells transfected with pGL2–190 were cultured in the presence of 125 nM TCDD, 0.2 mM or 2 mM phenobarbital for 16 h. A549 cells transfected with pGL2–651 were cultured in the presence of IL-1β (1 ng/ml) for 16 h. The bar graph represents mean ± S.E.M. from three experiments performed in duplicate. Data are expressed as relative change compared to unstimulated cells.

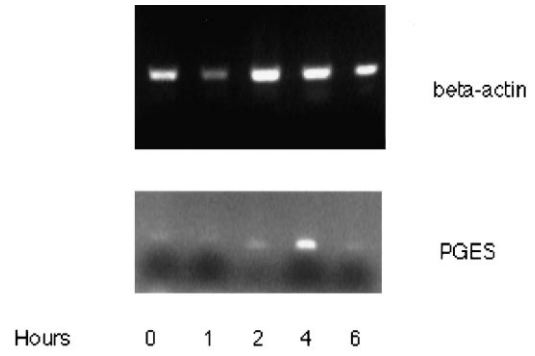

Fig. 3. IL-1β induction. RT-PCR analysis of PGES mRNA from A549 cells exposed to 1 ng/ml IL-1β for the indicated periods of time. The upper panel shows PCR products from β-actin mRNA, and the bottom panel shows PCR products generated from human PGES mRNA (exon 3 specific product 313 bp).

3.4. IL-1β induction of PGES mRNA expression

RT-PCR analysis showed that the transcript of PGES mRNA was stimulated by IL-1β in A549 cells (Fig. 3). An induction of mRNA was seen 4 h after exposure, followed by a decline. The same pattern was shown with transient transfections of A549 cells with the pGL2–651 reporter construct (Fig. 2B and data not shown).

3.5. Phenobarbital depresses the induction of PGES in A549 cells

A549 cells were treated with phenobarbital and/or IL-1β for 72 h. Microsomal fractions were prepared and analyzed for PGES expression by SDS–PAGE and Western blotting. Fig. 4 demonstrates that IL-1β induced PGES expression was blocked by treatment with phenobarbital. PGES activity measurements were consistent with the Western blot data (not shown).

4. Discussion

The PGES gene is located on chromosome 9q34.3, and it now appears that all known MAPEG members reside on different chromosomes. The gene structure of PGES is similar to its closest relative MGST1 with regards to exon/intron borders [4] and differs from other human MAPEG members, which consist of additional exons. Interestingly, the lipoca-

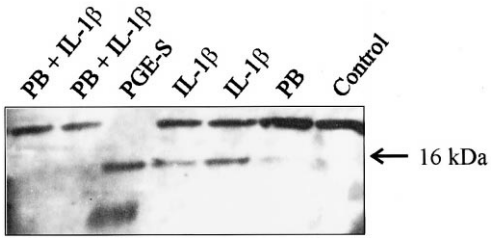

Fig. 4. Effect of phenobarbital on IL-1β induced PGES expression. Western blot analysis of PGES expression in microsomal fractions of A549 cells incubated with phenobarbital (PB) and/or IL-1β. As positive control (PGES), the membrane fraction from bacteria expressing human PGES was included (1 μg). In all other lanes, 10 μg of total protein was analyzed.

lin-type PGD synthase as well as COX-1 genes map to the same part of chromosome 9 as PGES, i.e. 9q34.2–q34.3 [18] and 9q32–33.3 [12,13], respectively. The putative promoter region of PGES is transcriptionally active and we have observed that reporter constructs are regulated by IL-1β and phenobarbital. In A549 cells, a transient rise in PGES mRNA and promoter activity was observed following exposure to IL-1β. The IL-1β induction of COX-2 mRNA as well as COX-2 promoter activity has been shown to follow a similar pattern [19].

Phenobarbital is a potent inducer of drug metabolizing enzymes, inhibits some tumor forms in humans, and is an effective anticonvulsant as well as sedative. The presence of a barbie box in the promoter led us to investigate the effect of phenobarbital on the expression of PGES. Interestingly, phenobarbital strongly reduced PGES promoter activity and induction by IL-1β of PGES protein. This effect may have broad implications in the context of a role of PGE_2 in sleep/wakefulness since PGD_2 and the lipocalin-type PGD synthase have been suggested to play a role in sedation and sleep [20]. In addition, PGE_2 has been reported to counteract the effect of PGD_2 [21] as well as to be present at higher levels during wakefulness [22]. A balance of these prostaglandins might thus control vital CNS functions. Interestingly, COX-2 activity has been shown to increase after phenobarbital treatment of Kupfer cells [23]. A possible mechanism for sleep induction by phenobarbital may therefore involve induction of COX-2 with down-regulation of PGES, thus leading to a shift from PGE_2 to PGD_2 biosynthesis.

In conclusion, here we report the first gene structure of a PGES gene. Our results and observations on regulatory properties form the basis for further research into the physiological function of PGES in health and disease.

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