Protein Kinase C Activates Human Lipocalin-type Prostaglandin D Synthase Gene Expression through De-repression of Notch-HES Signaling and Enhancement of AP-2β Function in Brain-derived TE671 Cells*

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Here we investigated the regulatory mechanism of lipocalin-type prostaglandin D synthase (L-PGDS) gene expression in human TE671 (medulloblastoma of cerebellum) cells. Reporter analysis of the promoter region from −730 to +75 of the human L-PGDS gene demonstrated that deletion or mutation of the N-box at −337 increased the promoter activity 220–300%. The N-box was bound by Hes-1, a mammalian homologue of Drosophila Hairy and enhancer of split, as examined by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. Functional expression of the Notch intracellular domain significantly increased Hes-1 expression and decreased L-PGDS expression level in TE671 cells. Moreover, knock-down of Hes-1 mRNA by RNA interference significantly enhanced the L-PGDS mRNA level, indicating that the L-PGDS gene expression is repressed by the Notch-Hes signaling. When the AP-2 element at −98 of the promoter region was deleted or mutated, the promoter activity drastically decreased to ~10% of normal. The AP-2 element was bound by AP-2β dominantly expressed in TE671 cells, according to the results of electrophoretic mobility shift assay and chromatin immunoprecipitation assay. L-PGDS expression was induced by 12-O-tetradecanoylphorbol-13-acetate in TE671 cells, and this induction was inhibited by a protein kinase C inhibitor. Stimulation of TE671 cells with 12-O-tetradecanoylphorbol-13-acetate or transfection with protein kinase Cα expression vector induced phosphorylation of Hes-1, inhibition of DNA binding of Hes-1 to the N-box, and activation of the AP-2β function to up-regulate L-PGDS gene expression. These results reveal a novel transcriptional regulatory mechanism responsible for the high level expression of the human L-PGDS gene in TE671 cells.

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS; EC 5.3.99.2, also known as β-trace; see Ref. 1) was originally purified from rat brain as the enzyme catalyzing the formation of PGD₂ from PGH₂, a common precursor of all prostanoids (2–5). L-PGDS is a secretory protein that is post-translationally glycosylated (6) and belongs to the lipocalin gene family (3). PGD₂ is a major prostanoid in the brain and has numerous physiological functions such as sleep regulation, pain responses, and inflammation (3, 7–9). Besides its function as a PGD₂-producing enzyme, L-PGDS also binds various lipophilic molecules, such as retinal, retinoic acid, biliverdin, and bilirubin, with high affinities (10, 11); and it is considered to act as a carrier protein for these compounds, like other proteins of the lipocalin gene family (3, 4, 12). Therefore, L-PGDS is a unique bifunctional protein acting as both PGD₂-producing enzyme and lipophilic molecule-binding protein.

Biochemical, physiological, and genetic properties of L-PGDS have been studied extensively in mammals (3–5). The L-PGDS gene has been isolated from human (13), mouse (14), and rat (15) species. L-PGDS mRNA is highly expressed in the heart (16), male genital organs (17–19), and the central nervous system (20–22). In the brain, L-PGDS is abundantly expressed in the leptomeninges, choroid plexus, and oligodendrocytes (20–22) but shows lower expression in neurons and astrocytes (23, 24). L-PGDS was identified as the second major gene product up-regulated in patients with multiple sclerosis (25) and is induced in stress protein αβ-crystallin-positive astrocytes in the active sclerotic plaques of these patients. In the gene-manipulated mice, human L-PGDS-overexpressing mice showed abnormality in the regulation of non-rapid eye movement sleep (26) and aggravation of the late phase allergic reactions occurring pathophysiologically in bronchial asthma (27). Moreover, studies using L-PGDS gene knock-out mice demonstrated that L-PGDS plays a critical role in the pain responses (14) and showed that such mice had an abnormal profile of non-rapid eye movement sleep after sleep deprivation (28). Recently, crystallization of recombinant mouse L-PGDS was successful (29), demonstrating that L-PGDS possesses a β-barrel structure consisting of eight β-strands and one α-helix that is highly conserved among the proteins of the lipocalin gene family (30).

There are several reports about the mechanisms regulating the transcription of the L-PGDS genes. For example, thyroid
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hormone activates L-PGDS gene expression through a thyroid hormone-responsive element in human TE671 cells derived from a cerebellar medulloblastoma (31). Dexamethasone induces L-PGDS gene transcription via glucocorticoid receptor in mouse neuronal GT1-7 cells (23). 17β-Estradiol regulates L-PGDS gene expression in a tissue-specific manner. In the heart, L-PGDS gene expression is activated through estrogen receptor β (32) and oppositely inhibited in the ventrolateral preoptic area, which is the critical area for PGD2-mediated systemic vasodilation (1.5%).

Recently, we found that rat L-PGDS gene expression is activated through estrogen receptor β in the rat primary cultured leptomenigeal cells (33). However, the physiological role of the Notch-Hes signaling in the regulation of L-PGDS gene expression and the mechanisms involved in high level expression of the L-PGDS gene in various types of cells are still unknown.

To approach the molecular mechanisms for the cell type-specific transcriptional regulation and high level expression of the human L-PGDS gene, we analyzed the expression of the human L-PGDS gene at the transcription level in TE671 cells. The N-box in the human L-PGDS gene promoter is a cell type-specific cis-element that is bound with the transcriptional repressor, Hes-1, in vivo as well as in vitro. Both the intracellular domain of Notch-1 (NICD) and Hes-1 significantly repressed L-PGDS gene expression. Moreover, knock-down of Hes-1 mRNA by RNA interference (RNAi) caused an increase in L-PGDS gene expression. We also found that the AP-2 element acts as a cis-element for transcriptional activation. Furthermore, 12-O-tetradecanoylphorbol-13-acetate (TPA)-activated protein kinase C (PKC) inhibits Hes-1 binding to the N-box by phosphorylation of Hes-1 and enhanced AP-2β function. PKC overcomes the Notch-Hes signaling-mediated repression and enhances AP-2β activity, thus resulting in high level expression of the human L-PGDS gene in TE671 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human TE671 (medulloblastoma) cells were kindly provided by Dr. David M. White (Chicago University, Chicago), and human 1321-N1 (astrocytoma) cells were a generous gift from Dr. Koichi Ishikawa (Gunma University, Maebashi, Japan). TE671, 1321-N1, and MCF-7 (breast cancer; American Type Culture Collection, Manassas, VA) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum under 5% CO2 atmosphere at 37 °C.

RNA Analysis—Preparation of total RNA and cDNA synthesis was carried out as described (34). PCR amplification was conducted by using ExTaq DNA polymerase (Takara Shuzo, Kyoto, Japan) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 28–35 cycles of 94 °C for 20 s, 55 °C for 20 s, and 74 °C for 30–90 s. Primers used in this study are described (35) and listed in Table I. The resultant PCR products were analyzed by electrophoresis in an agarose gel (1.5%).

Construction of Promoter-Luciferase Plasmids, Site-directed Mutagenesis, and Luciferase Assay—To define the cis-element(s) involved in the regulation of human L-PGDS gene expression in TE671 cells, we characterized the 5′-flanking region (−0.8 kb from −730 to +75) of the gene by conducting promoter luciferase reporter assays. The promoter regions were amplified by PCR with the gene-specific primer set with XhoI (sense) and HindIII (antisense) sites at their respective 5′-end and human genomic DNA as the template. The resultant PCR products were digested with XhoI and HindIII and then inserted into the upstream site of the luciferase reporter gene of the pGL3-Enhancer vector (Promega, Madison, WI). A fragment carrying the promoter region from −730 to +75 was cloned into a pGL3-Enhancer vector to construct −730/+75.” A deletion series was constructed in the same manner. Site-directed mutagenesis of the 5′-flanking region of the L-PGDS gene was performed as described previously (33). All constructs were subjected to nucleotide sequencing to verify the correct sequence and orientation. The cells were transfected with each promoter-luciferase reporter construct (0.3 µg) together with pRL-CMV (0.1 µg; Promega) carrying the Renilla luciferase gene under the control of the cytomegalovirus promoter by using Effectene (Qiagen, Hilden, Germany) or FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. The luciferase activity was measured by using a luciferase assay system (Promega). The reporter activity was calculated by normalizing the luciferase value with that of the Renilla luciferase control vector and expressed relative to that of the pGL3-Enhancer vector, which was defined as 1. All data were obtained from at least three independent experiments. The relative promoter activities were depicted as the mean ± S.D.

Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP) Assay—Nuclear extracts were prepared by the method of Dignam et al. (36). EMSA was carried out as described (35). Oligonucleotides used for EMSA are listed in Table I. ChIP assay was performed as described previously (33). Antibodies for Hes-1 and AP-2β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After immunoprecipitation and reverse cross-linking, the recovered DNA was used for PCR amplification with the gene-specific primer sets listed in Table I. PCR was conducted under the following conditions: initial denaturation at 95 °C for 3 min, followed by 28–34 cycles of 94 °C for 20 s, 55 °C for 20 s, and 74 °C for 30 s. The resultant PCR products were analyzed by electrophoresis on 2% (v/w) agarose gels.

RNAi-mediated Suppression of Hes-1 mRNA—Small interfering RNA was provided by a vector-based system using pSilencer1.0 vector (Ambion, Austin, TX). Small interfering RNA sequence was designed through the program of Ambion web-site (www.ambion.com). The oligonucleotides, 5′-GACACGATCTGACGATAGATGCGTCTG-3′ and 5′-AATTTAACAGACGATGCACTATCTGTCGTTT-3′, were annealed and heat-denatured at 90 °C for 15 min, and then stirred at room temperature for an additional 3 h. The duplex oligonucleotide was cloned into Apal/EcoRI-digested pSilencer 1.0 vector to obtain pSi-Hes-1. The cloned vector was subjected to nucleotide sequencing to verify the correct sequences. TE671 cells were transfected with the psi-HER1 vector (0.3 µg) with or without each of the −730/+75 or −730/+75 N-box mu constructs (0.1 µg). After 48 h of transfection, the luciferase reporter activity was assayed, and RNA was also isolated from the same transfected cells. Luciferase assay and RT-PCR analysis were carried out as described above.

Subcellular Fractionation—Cells were suspended in 10 mM Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM 2-mercaptoethanol, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 20 µg/ml pepstatin, and 1 mM phenylmethylene sulfonyl fluoride and disrupted by sonication. After centrifugation at 36,000 × g at 12,000 × g, the supernatants were recovered and centrifuged at 100,000 × g for 1 h. The resultant supernatants (cytosolic fraction) were collected. Pellets (membrane fraction) were resuspended in 10 mM Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 200 µg/ml leupeptin, 20 µg/ml aprotinin, 20 µg/ml pepstatin, and 1 mM phenylmethylene sulfonyl fluoride.

Western Blot Analysis—Cell lysates from different samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA). The membranes were incubated with specific primary antibodies, washed, and then incubated with second antibodies conjugated to horseradish peroxidase (Amersham Biosciences). Immunoreactive signals were detected by the use of ECL Western blotting Detection System (Amersham Biosciences) according to the manufacturer’s instruction.

In Vivo Phosphorylation and Immunoprecipitation—TE671 cells were transfected with the pcDNA3-Hes-1 vector carrying Hes-1 with the FLAG tag (37) (kindly gifted from Dr. Michael Caudy; Cornell University, New York). After 48 h of transfection, the cells were washed with phosphate-buffered saline and cultured for another 4 h in phosphate-free Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 0.5 µg/ml [32P]orthophosphate (Amersham Biosciences) in the presence or absence of TPA (100 ng/ml). Prior to TPA treatment, some cells were pretreated for 1 h with the PKC inhibitor bisindolylmaleimide I (Bia). Cells were lysed in 25 mM Tris–Cl, pH 7.5, containing 100 mM NaCl, 5 mM EDTA, 0.5% (v/v) Triton X-100, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 20 µg/ml pepstatin, 50 mM Na2MoO4, 1 mM NaF, 1 mM phenylmethylene sulfonyl fluoride. After centrifugation for 30 min at 12,000 × g, the supernatants were collected.

Cell lysates were incubated with anti-FLAG monoclonal antibody conjugated to Sigma (Sigma) for 4 h at 4 °C with continuous agitation. After centrifugation, the pellets were washed five times with 10 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 0.1% (v/v) Triton X-100. The pellets were then suspended in SDS loading buffer, after which the proteins were separated by SDS-PAGE and analyzed with a Fluores-
RESULTS

Cell Type-specific Expression of Human L-PGDS Gene—The expression of the L-PGDS gene was investigated in various human cell lines by RT-PCR analysis. Human L-PGDS mRNA was highly expressed in TE671 cells, weakly in MCF-7 cells, and negligibly in 1321-N1 cells (Fig. 1A, upper panel), whereas the expression level of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, as the internal control, was almost identical in these three types of cells (Fig. 1A, lower panel). This result indicated that human L-PGDS gene expression was regulated in a cell type-specific manner.

To confirm the cell type-specific expression of the L-PGDS gene, we transiently transfected TE671, MCF-7, and 1321-N1 cells with the luciferase-reporter constructs containing the −730/+75 promoter region of the human L-PGDS gene. When the −730/+75 or −320/+75 construct was used for the transfection, efficient reporter activity was detected only in TE671 cells but not detected in MCF-7 or 1321-N1 cells (Fig. 1B). This result is therefore consistent with that of the expression analysis by RT-PCR (Fig. 1A). No efficient reporter activities were detected in any of the cells transfected with the −90/+75 construct. Therefore, these results indicate that the region from −730 to −90 contained the cis-elements responsible for the cell type-specific regulation of human L-PGDS gene expression in TE671 cells.

Analysis of Promoter Region of Human L-PGDS Gene—To identify the cis-acting element(s) responsible for transcriptional regulation of the human L-PGDS gene, we constructed serially deleted promoter-luciferase reporter plasmids containing various lengths of the promoter region of the human L-PGDS gene. TE671 cells were transfected with each construct, and the luciferase reporter activities were then measured. When the −730/+75 construct was used for the transfection, efficient reporter activity was detected (Figs. 1B and 2A). Deletion of the region from −730 to −400 did not result in any significant change in the promoter activity. On the contrary, further deletion to −320 caused a strong increase in the promoter activity, which was ~300% of that of the −730/+75 construct, indicating that this region from −400 to −320 contained a critical cis-element for the transcriptional repression. Further deletion from −320 to −105 did not show any change in the reporter activity (Fig. 2A). Deletion down to −90 resulted in a strong loss of reporter activity, to ~10% of that of the −730/+75 construct. Further deletion to −50 with the −50/+75 construct showed the weak reporter activity that is almost identical to that of the −90/+75 construct, indicating that the region from −105 to −90 contains the cis-element responsible for the transcriptional activation (Figs. 1B and 2A). Thus, these results indicate that cis-elements critical for the transcriptional regulation of the human L-PGDS gene were located in the regions from −400 to −320 and −105 to −90.

The initial deletion analysis demonstrated that critical cis-elements were localized within the proximal promoter region from −400 to −90. Various putative transcription factor-binding elements, such as the N-box at −337, GATA element at −288, krüppel-like factor-3-binding element at −247, and AP-2 element at −98, were found by analyzing the proximal promoter region by use of MatInspector (39).

To localize further the cis-acting elements in the proximal promoter region of the human L-PGDS gene, more detailed deletion and mutation analyses were carried out (Fig. 2B). When the region from −400 to −350 was deleted, the promoter activity was not altered. On the contrary, deletion of the region from −350 to −320 led to significant enhancement of the promoter activity, indicating that the cis-element responsible for transcriptional repression was present in the region from −350 to −320. This region contains the N-box consensus sequence at −337. Site-directed mutation of the N-box was done to obtain the −730/+75 N-box mu and −400/+75 N-box mu constructs. Reporter activities of these mutant constructs were increased ~220% when compared with the activity of the −400/+75 construct. The induction rate was almost identical when the region from −350 to −320 was deleted (~300%). These results indicate that human L-PGDS gene expression was repressed through the N-box in TE671 cells.

The reporter activity was not changed by deletion of the region from −320 to −105 containing the GATA element and the krüppel-like factor-3-binding element. Further deletion of the promoter between −105 and −90 caused a dramatic decrease in the reporter activity, indicating that the cis-element responsible for transcriptional activation resided in the region from −105 to −90. This region contains the AP-2 element starting at −98. When the −730/+75 AP-2 mu and −400/+75 AP-2 mu construct carrying the mutation at the AP-2 element was used to transfect TE671 cells, the reporter activity was decreased ~10% as compared with that of the −400/+75 construct, indicating that the AP-2 element at −98 functions for the transcriptional activation of human L-PGDS gene expression.

Taken together, the results presented thus far indicate that transcription of the human L-PGDS gene was repressed through the N-box and activated via the AP-2 element in TE671 cells.

Binding of Transcription Factor to N-box—Next we investigated the binding of nuclear factors to the N-box by conducting EMSA and ChIP assay. A radiolabeled double-stranded oligonucleotide (Table I) was used for EMSA along with the nuclear
extracts prepared from TE671 cells. A shifted band was observed when the nuclear extracts prepared from TE671 cells were added (Fig. 3A, lane 2). On the contrary, no corresponding shifted band was detected when the nuclear extracts were not added (Fig. 3A, lane 1). This shifted band disappeared when excess amounts of the unlabeled probe (10- or 50-fold) were added (Fig. 3A, lanes 3 and 4), whereas the band intensity was not altered by the addition of an excess amount of the unlabeled probe carrying a mutation in the N-box (Fig. 3A, lane 5).

These results indicate that some nuclear factors bound to the N-box in vitro. The N-box is known as the target element of the Hes family proteins (40). So we carried out RT-PCR analysis to investigate the expression profile of Hes family proteins in TE671 cells (Fig. 3B). Hes-1 was highly expressed in these cells, whereas expressions of Hes-3 and -5 were under the detection limit of our experimental conditions. Therefore, we suspect that the N-box was bound by Hes-1 in TE671 cells. In addition, Notch-1, which is involved in the Notch-Hes signaling pathway, was abundantly expressed in TE671 cells (Fig. 3B). To confirm whether Hes-1 binds to the N-box, we performed a supershift assay using anti-Hes-1 antibody. The shifted signal was observed when the nuclear extracts were used (Fig. 3C, lane 1). This DNA-protein complex migrated more slowly by addition of

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**Table I**

| Oligonucleotide sequences used in this study |
|---------------------------------------------|
| For PCR                                     |
| L-PGDS 5'-GACACCGAGGCCCCAGGGTC-3' and 5'-GGTGTTCGGCTATGCCACTTA-3' |
| G3PDH 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACACCCCTGTTGCATGA-3' |
| Hes-1 5'-ATGCCAGCTGTATATAATTGGAG-3' and 5'-TGGCGGTGCCCAGGGATGTCATGG-3' |
| Hes-3 5'-ATGGAGAAGAGGCGTGTCGCCG-3' and 5'-TCACACGGCCGGCCACAGCGAAC-3' |
| Notch-1 5'-GATGGCTCAATGGGAGTGGCTG-3' and 5'-GGGAGGAGTGCTGTCG-3' |
| For ChIP assay                              |
| N-box 5'-GCTGGGGCGTTTCAAGGACAAAAC-3' and 5'-GGGAGAGGGTGTTCTCAGGC-3' |
| AP-2 5'-TGCTTCAGCTGGCCAGCAGC-3' and 5'-GGCCACTGAACACTCGTG-3' |
| For EMSA                                    |
| N-box 5'-GCCCCAGTGGGAGTTGGCGGCCGAGGCA-3' |
| N-box mu 5'-GGCCCCAGTGGGAGTTGGCGGCCGAGGCA-3' |
| AP-2 5'-AGCAGTGGCTGCCCCCTGACCAGCGATCAGGTA-3' |
| AP-2 WT 5'-GATCGAACAGCGCCGAGGCGCC-3' |
| AP-2 mu 5'-GATCGAACAGCGCCGAGGCGCC-3' |

* Mutated nucleotides are underlined.

* These oligonucleotides were purchased from Promega.
amplification as the input control (Input). Results are representative of three independent experiments.

Unlabeled oligonucleotides were added as the competitor. NS means nonspecific signal. B, expression of Hes isoforms and Notch-1 in TE671 cells. Total RNA prepared from TE671 cells was used for RT-PCR analysis. PCR cycling was performed for 35 cycles. Resultant PCR products were separated and detected by agarose gel electrophoresis. Nuclear extracts prepared from TE671 cells were preincubated with anti-Hes-1 antibody for 30 min at room temperature. Then the mixture was incubated with radiolabeled oligonucleotide containing the N-box, and EMSA was performed as described above. C, supershift assay. Nuclear extracts prepared from TE671 cells were preincubated with anti-Hes-1 antibody for 30 min at room temperature. Then the mixture was incubated with radiolabeled oligonucleotide containing the N-box, and EMSA was performed as described above. D, scheme for ChIP assay of the N-box. Amplicon containing the N-box was 157 bp. E, in vivo binding of Hes-1 to the N-box of the human L-PGDS gene promoter. TE671 cells transfected with empty vector (pcDNA3.1), Hes-1, or NICD were treated with formaldehyde to cross-link DNA and proteins. The DNA-protein complexes were immunoprecipitated (IP) by the anti-Hes-1 antibody, and the extracted DNA was used as the template for PCR amplification (35 cycles). L-PGDS N-box means amplification of the N-box of the L-PGDS promoter; no N-box indicates that the amplified region does not contain the N-box. A small aliquot before immunoprecipitation was used for PCR amplification as the input control (Input). Results are representative of three independent experiments.

anti-Hes-1 antibody to the reaction mixture (Fig. 3C, lane 2). These results suggest that Hes-1 bound to the N-box of the human L-PGDS promoter in vitro.

To know whether Hes-1 binds to the N-box in vivo, we carried out the ChIP assay. TE671 cells were treated with formaldehyde, and then the chromatin-DNA complex was immunoprecipitated by anti-Hes-1 antibody. The purified precipitates were analyzed by PCR with the specific primer sets. The PCR primers specific for L-PGDS promoter produced an amplicon of predicted size (157 bp; Fig. 3D) from both total input DNA and immunoprecipitates obtained with the anti-Hes-1 antibody (Fig. 3E). On the other hand, the primers for the region without the N-box consensus sequence did not produce an amplicon. Moreover, the signal intensity of amplification was significantly increased when TE671 cells were transfected with the pcDNA3-Hes-1 vector carrying Hes-1 or the pcDNA3-cytohN1 vector carrying NICD (41) (kindly provided from Dr. Artavanis-Tsakonas, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA). These results indicate that Hes-1 binds to the N-box of L-PGDS promoter region in vivo as well as in vitro. In addition, enhancement of the intracellular Hes-1 level through overexpression by pcDNA3-Hes-1 or pcDNA3-cytohN1 vector increased the Hes-1 binding ability to the N-box of the L-PGDS gene promoter in vivo.

Notch-Hes Signal Suppresses L-PGDS Expression—We then investigated the roles of the Notch-Hes signaling in the regulation of human L-PGDS expression. When TE671 cells were transfected with the pcDNA3-cytohN1 vector, both the mRNA and protein levels of Hes-1 were clearly increased as compared to when the empty vector (pcDNA3.1) was transfected (Fig. 4A). Reversely, transfection of TE671 cells with each pcDNA3-Hes-1 or pcDNA3-cytohN1 vector significantly decreased L-PGDS in both mRNA and protein levels (Fig. 4B). To elucidate whether Hes-1 or NICD-mediated repression of L-PGDS expression occurs through the N-box, we co-transfected TE671 cells with each pcDNA3-Hes-1 or pcDNA3-cytohN1 vector together with the −730/+75 or −730/+75 N-box mu construct. Co-transfection experiments demonstrated that the reporter activity was decreased (Fig. 4C) in an intracellular Hes-1 level-dependent manner (Fig. 4C, inset). However, when the cells were transfected with the −730/+75 N-box mu construct, the reporter activity was not altered (Fig. 4C) even if the Hes-1 was overexpressed (Fig. 4C, inset). Renilla luciferase activities from pRL-CMV vector as an internal control were not changed in any cases. These results indicate that NICD and Hes-1 involved in the Notch-Hes signaling repressed human L-PGDS gene expression through the N-box in TE671 cells.

Next, we used RNAi technology to perform a knock-down study on Hes-1 mRNA in TE671 cells. Vector-based RNAi for Hes-1 decreased the endogenous Hes-1 mRNA level in TE671 cells (Fig. 4D, upper panel). The same cells, L-PGDS gene expression was significantly induced by the knock-down of Hes-1 mRNA (Fig. 4D, middle panel), whereas the G3PDH mRNA level was not altered (Fig. 4D, lower panel). TE671 cells were co-transfected with either pSi-Hes-1 or pSi-empty (pSi-lencer1.0) vector together with the −730/+75 construct. The reporter activity obtained with the pSi-Hes-1 vector was increased about 2-fold as compared with that obtained when the
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Fig. 4. Function of Notch-Hes signaling in the regulation of L-PGDS gene transcription. A, overexpression of NICD increased both mRNA and protein levels of Hes-1 in TE671 cells. B, overexpression of Hes-1 and NICD decreased L-PGDS mRNA and protein levels in TE671 cells. Total RNA was prepared from transfected cells and utilized for subsequent RT-PCR analysis. The L-PGDS protein levels were detected by horseradish peroxidase-conjugated anti-L-PGDS monoclonal antibody (Ab) (1B7) (42). C, suppression of L-PGDS gene expression by Hes-1 or NICD. TE671 cells were co-transfected with the −730/+/75 or −730/+/75 N-box mu construct (300 ng) and 0, 50, and 100 ng of each of pcDNA3-Hes-1 or pcDNA3-cytohN1 vector. Total plasmid quantity was adjusted by adding of empty pcDNA3.1 vector to 100 ng. Inset, Hes-1 gene expression was investigated by RT-PCR when the cells were transfected with each amount of the expression vector. The firefly and Renilla luciferase activities were measured after 48 h of transfection. The reporter activity of the −730/+/75 construct was defined as 100%. Firefly and Renilla luciferase activities are indicated by solid and shaded columns, respectively. D, RNAi for Hes-1 mRNA increased L-PGDS gene expression. TE671 cells were transfected with either the pSi-Hes-1 vector or its empty vector (0.4 μg). RNA was prepared from each cell 40 h after the transfection, and RT-PCR analysis was performed to measure Hes-1 and L-PGDS mRNA levels. G3PDH was used as the internal control. E, knock-down of Hes-1 mRNA by RNAi enhanced L-PGDS promoter activity through the N-box. The reporter assay of the human L-PGDS promoter demonstrated that when TE671 cells transfected with the −730/+/75 construct was used for the transfection (Fig. 4E). On the contrary, when the −730/+/75 N-box mu construct was utilized for co-transfection with either pSi-Hes-1 or pSi-empty vector, the reporter activity was enhanced to a level almost the same as that when the cells were transfected with both the −730/+/75 construct and the pSi-Hes-1 vectors. These results indicate that the intracellular Hes-1 level directly affected L-PGDS gene expression through the N-box in TE671 cells.

Analysis of Binding of Nuclear Factor to AP-2 Element—Binding between nuclear factors and the AP-2 element was investigated by EMSA and the ChIP assay. For EMSA, the DNA probe containing the AP-2 element was used. The specific DNA-protein binding activity was detected with nuclear proteins prepared from TE671 cells (Fig. 5A, lane 2). On the other hand, the binding activity to the probe was undetectable when the nuclear extracts were not added (Fig. 5A, lane 1). The detected signal did not appear with an excess amount of the unlabeled AP-2 probe or authentic AP-2 wild-type (WT) probe (Fig. 5A, lanes 3–5), but DNA-protein complex formation was not inhibited in the presence of an excess amount of the mutated AP-2 mu probe (lane 6).

To prove the in vivo binding of the nuclear factor to the AP-2 element, we performed the ChIP assay. First of all, we examined the expression profile of the AP-2 subtypes, α, β, and γ, in TE671 cells by RT-PCR with subtype-specific primers (35). AP-2β was dominantly expressed, and AP-2α and γ were detected in negligible amounts in TE671 cells (Fig. 5B). In the supershift assay, the DNA-protein complex was supershifted by the addition of anti-AP-2β antibody (Fig. 5C, lane 2) as compared to when the nuclear extracts prepared from the TE671 cells were used in the absence of anti-AP-2β antibody (Fig. 5C, lane 1). Furthermore, we carried out in vivo binding analysis by the ChIP assay with anti-AP-2β antibody. The expected size (112 bp) of an amplicon containing the AP-2 element at −98 was detected in the formaldehyde-mediated DNA-protein complexes immunoprecipitated with anti-AP-2β antibody (Fig. 5, D and E). On the other hand, there was no detectable signal when the antibody was not added, although the signals were detected in both cases for the input control. There was no signal when the region containing no AP-2 element was amplified. These results indicate that AP-2β binds to the AP-2 element of the L-PGDS promoter both in vitro and in vivo.

TPA Enhances L-PGDS Expression Level—TPA is known to be an activator for PKC signaling and to activate AP-2 function (43). L-PGDS expression was enhanced ∼2.5-fold by the treatment with TPA as compared with the expression obtained with the vehicle control (Fig. 6A). This TPA-mediated induction of L-PGDS was nullified by co-treatment with Bis, a PKC inhibitor. The reporter assay of the human L-PGDS promoter demonstrated that when TE671 cells transfected with the −730/+/75 construct was used for the transfection (Fig. 6E). On the contrary, when the −730/+/75 N-box mu construct was utilized for co-transfection with either pSi-Hes-1 or pSi-empty vector, the reporter activity was enhanced to a level almost the same as that when the cells were transfected with both the −730/+/75 construct and the pSi-Hes-1 vectors. These results indicate that the intracellular Hes-1 level directly affected L-PGDS gene expression through the N-box in TE671 cells.
+75 construct were treated with TPA, the reporter activity was enhanced ~2.5-fold as compared with that of the vehicle control (Fig. 6B). This induction was not observed when Bis was added prior to the TPA treatment, being consistent with the results of expression analysis (Fig. 6A). Reporter activity of the −105/+75 construct was enhanced ~1.4-fold as compared with that of the vehicle control. Further deletion of the region containing the AP-2 element (the −90/+75 construct) showed a
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drastic decrease in the promoter activity as well as in the TPA responsiveness. These results suggest that the AP-2 element at −98 is the TPA-responsive element. Most interestingly, the induction level by TPA for the −730/+75 construct (−2.5-fold) was higher than that for the −105/+75 construct (−1.4-fold), indicating that some other TPA-responsive element(s) exist within the region from −730 to −105 of the human L-PGDS promoter.

A previous report (44) indicated that the DNA-binding domain of Hes-1 was phosphorylated by PKC and that phosphorylated Hes-1 lost its DNA-binding ability. At first, we investigated whether Hes-1 in TE671 cells is phosphorylated by PKC. We incubated the pcDNA3-Hes-1 (FLAG) vector-transfected TE671 cells with [32P]orthophosphate, and we used the cell lysates for subsequent immunoprecipitation analysis with anti-FLAG antibody. The intensity of the radiolabeled signal in TPA-treated cells was significantly higher than that in non-treated cells, and its intensity was decreased by the co-treatment of TPA and Bis, although the precipitates reacted in the same intensity with either anti-Hes-1 or anti-FLAG antibody in both samples (Fig. 6C). Next, we investigated the localization of PKC by using the anti-phospho-PKCα/β antibody when TE671 cells were treated with TPA. PKC was translocated from the cytosol to the membrane fraction in a time-dependent manner by the treatment of TE671 cells with TPA (Fig. 6D).

Induction of L-PGDS by PKC Activation—We investigated the roles of the PKC activation in the transcriptional regulation of the L-PGDS gene in TE671 cells. When PKCα was expressed heterologously in TE671 cells, the L-PGDS level was significantly increased (Fig. 7A). When TE671 cells were transfected with the −730/+75 construct with FLAG-ca.PKCα vector, the reporter activity was enhanced −2-fold as compared to when both the −730/+75 construct and the FLAG-empty (pFLAG-CMV2) vector were co-transfected (Fig. 7B).

EMSA demonstrated that binding of Hes-1 to the N-box was decreased by the treatment with TPA (Fig. 7C). However, by the treatment with Bis prior to the TPA treatment, the binding ability of Hes-1 to the N-box was restored. Moreover, the expression of PKCα decreased the binding ability of Hes-1 to the N-box (Fig. 7C). The results of the ChIP assay with anti-Hes-1 antibody showed that there is no amplification signal when TPA treatment enhanced the nuclear protein induction level by TPA for the 98 is the TPA-responsive element. Most interestingly, the knock-down of Hes-1 mRNA with Hes-1 RNAi (Fig. 4). All these data clearly

**FIG. 7.** PKC enhances L-PGDS expression. A heterologous expression of FLAG-ca.PKCα and its empty vector (FLAG-empty, pFLAG-CMV2) in TE671 cells. Each transfectant was disrupted, and crude extracts were used for immunoblot analysis with anti-FLAG antibody or anti-L-PGDS antibody. B, TE671 cells were transfected with FLAG-empty or FLAG-ca.PKCα vector (0.3 µg) with the −730/+75 construct (0.1 µg). After 48 h, the luciferase activities were measured as described under “Experimental Procedures.” C, EMSA using nuclear extracts prepared from TE671 cells treated with TPA in the presence or absence of Bis or expressed with PKCα. Detected signal is indicated by the arrow. D, in vivo binding analysis of Hes-1 to the N-box in TE671 cells. TE671 cells treated or not with TPA and/or Bis, or expressed with PKCα, were used for the ChIP assay. A small aliquot before immunoprecipitation (IP) was used for PCR amplification (35 cycles) as the input control (Input). Results are representative of three independent experiments. E, EMSA for the AP-2 element as described in C. Detected signal is indicated by the arrow. F, ChIP assay for AP-2 binding to the AP-2 element. TE671 cells treated with the indicated chemicals or expressed with PKCα were used for the ChIP assay. Cross-linked DNA-protein complexes were precipitated with anti-AP-2β antibody and then used for PCR amplification (35 cycles) after reverse cross-linking. A small aliquot before immunoprecipitation (IP) was used for PCR amplification as the input control (Input). Results are representative of three independent experiments.

**DISCUSSION**

In a previous study, we demonstrated that rat L-PGDS gene expression was regulated by Hes-1 and interleukin-1β in the primary cultured leptomeningeal cells (33). Another group (31) analyzed the human L-PGDS gene promoter and identified a thyroid hormone-responsive element in it. In that study, deletion of the region from −595 to −325 was shown to enhance the promoter activity in TE671 cells, suggesting that this region contains negative cis-element(s) that inhibit L-PGDS gene expression. However, the cis-element was not identified. In this study, we found a novel transcriptional regulatory mechanism composed of the Notch-Hes signaling and AP-2 activation of the L-PGDS gene in TE671 cells. Furthermore, we also showed that PKC-mediated de-repression of the Notch-Hes signaling and AP-2 activation are responsible for the high level expression of the human L-PGDS gene found in these cells. Fig. 8 summarizes the proposed transcription regulatory mechanism of the human L-PGDS gene in TE671 cells.

The N-box sequence, CACCAG, at −337 of the human L-PGDS gene promoter is identical to the N-box consensus sequence CACNAG (40). We demonstrated here that the N-box was essential for repression of the L-PGDS gene expression, by deletion or site-directed mutation analysis (Figs. 1 and 2), by EMSA and ChIP assay (Figs. 3 and 4), by functional assays involving co-expression of NICD or Hes-1, and by knock-down of Hes-1 mRNA with Hes-1 RNAi (Fig. 4). All these data clearly
Our present study provides a novel transcriptional regulatory mechanism for high level expression of the human L-PGDS gene, one mediated by Notch-Hes signaling and AP-2 activation. We also showed that this expression was enhanced by TPA treatment and that TPA-mediated enhancement occurred through activation of the PKC signal, because the PKC inhibitor BIS abolished TPA-mediated transcriptional activation of the human L-PGDS gene (Fig. 6). Moreover, PKC-mediated L-PGDS gene expression through inhibition of DNA binding of Hes-1 to the N-box and enhancement of binding ability of AP-2β to the AP-2 element (Fig. 7). In the pathway of prostaglandin biosynthesis, only cyclooxygenase-2, an enzyme acting upstream of L-PGDS, is transcriptionally up-regulated 2-3-fold by PKC in rat intestinal epithelial RIE-1 cells (53). The cyclooxygenase-2 gene promoter contains the E-box, but mutation of the E-box decreased or did not affect the promoter activity in various cells (54–56). Therefore, our proposed novel mechanism for the regulation of the human L-PGDS promoter by the Notch-Hes signaling is a unique one in prostaglandin biosynthesis.

We propose the following transcriptional regulation of the L-PGDS gene by PKC (Fig. 8). In untreated cells, L-PGDS gene expression is repressed through the N-box by the Notch-Hes signal and activated via the AP-2 element by AP-2β. Thus, L-PGDS is only moderately expressed. Once the PKC signal is activated, the repression by the Notch-Hes signal is abrogated by phosphorylation of Hes-1 DNA-binding domain, which blocks its binding to the N-box. Moreover, PKC can activate AP-2 function by enhancing the ability of AP-2β to bind to the AP-2 element. Therefore, PKC functions as an activator for high level expression of the L-PGDS gene in brain-derived TE671 cells.

Clinical studies showed the importance of many lipocalin gene family proteins including the L-PGDS (12). Actually, the L-PGDS level is enhanced in serum or cerebrospinal fluid of the patients with hypertension (57), arteriosclerosis (16), or subarachnoid hemorrhage (58). Thus, L-PGDS is thought to be a useful as the diagnostic marker for those diseases (3, 4). However, the mechanisms responsible for enhancement of the L-PGDS level associated with such diseases have never been identified. High pressure significantly increases the phosphorylation of PKC in arteries (59), and the plasma L-PGDS level is increased in the coronary artery with arteriosclerosis (16).

Low density lipoprotein related to the progression of arteriosclerosis can activate PKC in vascular smooth muscle cells (60), in which the L-PGDS expresses (16, 61) and functions in the Notch signal (62). Moreover, PKC is activated in a variety of animal models of vasospasm after subarachnoid hemorrhage (63). L-PGDS is secreted into the human cerebrospinal fluid from leptomeningeal cells (64), and in these cells, L-PGDS gene expression was repressed by Hes-1, as we showed in a previous study (33). Therefore, the present findings together with these earlier results indicate that the increase in the L-PGDS level in patients with the above diseases might, at least in part, occur through PKC-mediated de-repression of the Notch-Hes signal and enhancement of AP-2β activation.

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Protein Kinase C Activates Human Lipocalin-type Prostaglandin D Synthase Gene Expression through De-repression of Notch-HES Signaling and Enhancement of AP-2β Function in Brain-derived TE671 Cells

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