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Mechanism of Increased PLD1 Gene Expression During Early Adipocyte Differentiation Process of Mouse Cell Line 3T3-L1

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ABSTRACT

A mouse cell line 3T3-L1 is differentiated into adipocytes when treated with an inducer cocktail (IDX) (insulin, dexametahsone, and a cAMP phosphodiesterase inhibitor of isobutyl-methylxanthine (IBMX)). Here, we report that *PLD1*, but not *PLD2*, mRNA and protein increased during the early differentiation process. Our analysis shows that IDX resulted in a sequential induction of C/EBPβ, PLD1, and C/EBPα which is a key transcription factor of late adipocyte differentiation. Among the three inducers, IBMX + any other inducer induced mild adipocyte differentiation, whereas insulin + dexamethasone did not. IBMX increased *PLD1* but not *PLD2* mRNA. Forskolin, an adenylate cyclase activator, and dbcAMP also increased *PLD1* mRNA, suggesting the cellular cAMP as the inducer of both adipocyte differentiation and *PLD1* transcription. We focused on the regulatory mechanism of *PLD1* transcription during this differentiation process. IDX or a combination of inducers including IBMX increased *PLD1* promoter activity, which is consistent with mRNA analysis. Promoter analysis identified two adjacent C/EBP motifs located between −338 and −231 bp from the first exon as the IBMX responsive elements. Furthermore, overexpression of C/EBPβ, but not C/EBPα, increased *PLD1* mRNA and *PLD1* 5′ promoter activity. EMSA and chromatin immunoprecipitation assay confirmed the direct binding of C/EBPβ, but not C/EBPα, to these C/EBP motifs of *PLD1* 5′ promoter. Our results show that PLD1 is a target gene of C/EBPβ through the increased cellular cAMP during early adipocyte differentiation of 3T3-L1 cells. J. Cell. Biochem. 109: 375–382, 2010.

KEY WORDS: ADIPOCYTE DIFFERENTIATION; PLD1 GENE EXPRESSION; IBMX; C/EBP; PROMOTER ANALYSIS; EMSA; ChIP ASSAY

Excessive accumulation of triglycerides in lipid droplets is associated with metabolic disorders such as insulin resistance and type 2 diabetes [Browning and Horton, 2004]. The understanding of adipogenesis is an urgent requirement for therapeutic intervention in metabolic diseases such as obesity and atherosclerosis.

Phospholipse D (PLD) is an enzyme that catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PLD is widely distributed in a variety of species [Nozawa, 2002]. In mammalian cells, PLD is activated by various extracellular stimuli and plays an important role in signal transduction, leading to rapid responses such as secretion, cytoskeletal rearrangement, and long-term responses including proliferation, differentiation, apoptosis, and survival. PLD activity is regulated by various factors including the Arf family, Rho family of the small G protein, and protein kinase C (PKC) [Exton, 2002]. Although the mechanism of PLD activation has been extensively studied, reports analyzing the mRNA level of PLD are scarce [Yoshimura

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et al., 1996; Nakashima et al., 1998; Kikuchi et al., 2007; Kang et al., 2008].

Using a cell-free system, it has been reported that PLD is important for the assembly of very low-density lipoproteins, which appears to involve the formation of a lipid droplet in the microsomal lumen [Marchesan et al., 2003]. Among the two PLD isoforms, PLD1 is known to be involved in oleic acid-induced adipocyte differentiation of NIH3T3 cells [Nakamura et al., 2005; Andersson et al., 2006]. Exogenous PA was shown to stimulate lipid droplet formation [Marchesan et al., 2003]. PLD1 was also observed to regulate insulin-stimulated plasma membrane fusion of Glut4 glucose transporter-containing vesicles [Huang et al., 2005].

These studies analyzing PLD in adipocyte differentiation were performed either by transfecting expression vector or by incubating cells with oleic acid to increase the basal production of lipid droplet. Since *PLD* gene expression during the adipocyte differentiation process has not been precisely analyzed, examination of the mechanism of PLD transcription would provide useful information for better understanding the role of PLD in the adipogenetic process. Preadipocyte cell line such as 3T3-L1 has been frequently used as a model of adipocyte differentiation when treated with appropriate inducers [Brun et al., 1996; Spiegelman and Flier, 1996]. In the current study, we analyzed two *PLD* isoform gene expressions in 3T3-L1 cells treated with a cocktail of three inducers (IDX) (insulin, dexamethasone, and isobutyl-methylxanthine (IBMX)). *PLD1*, but not *PLD2*, mRNA was found to increase during the early phase of differentiation inducted by IDX, and Forskolin, dbcAMP as well as IBMX could increase *PLD1* mRNA, suggesting the important role of intracellular cAMP. Furthermore, it was revealed that C/EBPβ, an important transcription factor for the early adipocyte differentiation process, directly increased *PLD1* gene expression.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

NIH3T3, HEK293, and mouse preadipocyte 3T3-L1 cells (ATCC CL173) were used. pcDNA 3.1 (+) human C/EBPα and human C/EBPβ expression vectors were generously provided by Dr. H. Hirai (Kyoto Prefectural University of Medicine, Kyoto, Japan). Forskolin and dibutylic cAMP (dbcAMP) were purchased from Sigma (St. Louis, MO) Anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ, and anti-PPARγ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

ADIPOCYTE DIFFERENTIATION INDUCTION

3T3-L1 cells were cultured in a growth medium (DMEM containing 10% calf serum) until confluence. Two days after reaching confluence (day 0), differentiation was induced by incubation of the cells in the differentiation medium containing 10 μg/ml insulin (Ins) (Sigma), 1 μM dexamethasone (Dex) (Sigma), and 0.5 mM IBMX (Sigma) (abbreviated as IDX). After 2 days, the media were replaced with 10% FCS in DMEM and the culture was continued to day 7. In some experiments, single or combinations of two inducers were used to examine adipocyte differentiation.

OIL-RED-O-STAINING

Oil-Red-O (0.4%) in isopropanol was diluted with two volumes of water, filtered and incubated with 3T3-L1 cells for 30 min at room temperature after fixing cells with 10% formaldehyde in PBS for 30 min. The cell monolayer was washed with water once and the stained triglyceride droplets within the cells were visualized under a microscopy.

SEMI-QUANTITATIVE RT-PCR

Semi-quantitative RT-PCR of mouse *PLD1*, *PLD2*, *matrix metalloproteinase-9* (*MMP-9*), *tissue inhibitor of matrix metalloproteinase 1* (*TIMP-1*), and *β-actin* was performed with the primer sets described below. In preliminary experiments, suitable amounts of cDNA and the range of PCR cycles that permit the linear amplification of each mRNA were determined. The primer sets used were the following:

Mouse *PLD1* primers: forward, 5′-CGTCCCCGCCAAAGTGCAG-3′; and reverse, 5′-CCGATATCTCTGGCCTTCCCTGT-3′.
Mouse *PLD2* primers: forward, 5′-TGCTCCCTTTGGCTCGCTTT-3′; and reverse, 5′-GGATCACCCCTTCCAGTCCTTT-3′.
Mouse *MMP-9* primers: forward, 5′-AGGGTTTTCTTCTTCTCTGGAC-3′; and reverse, 5′-AGGCACGCCCCTTGCTGA-3′.
Mouse *TIMP-1* primers: forward, 5′-TGCAACTCGGACCTGGTCA-3′; and reverse, 5′-GGGCATATCCACAGAGGTTT-3′.
Mouse *β-actin* primers: forward, 5′-CCGTGAAAAGATGACCCAGA-3′; and reverse, 5′-GTCTCCGGAGTCCATCACAA-3′.

The PCR conditions for mouse *PLD1*, *PLD2*, *MMP-9*, *TIMP-1*, and *β-actin* were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The numbers of cycles of mouse *PLD1*, *PLD2*, *MMP-9*, *TIMP-1*, and *β-actin* were 28, 30, and 32; 28, 30, and 32; 30, 32, and 34; 28, 30, and 32; and 20, 22, and 24, respectively. Band intensities of mouse *PLD1* at 28 cycles, mouse *PLD2* at 30 cycles, *MMP-9* at 32 cycles, *TIMP-1* at 30 cycles, and mouse *β-actin* at 22 cycles were measured by NIH image version 6. The relative expression levels of each mRNA were normalized by *β-actin mRNA* expression level.

PREPARATION OF REPORTER VECTORS CONTAINING VARIOUS *PLD1* 5′ PROMOTER REGIONS

We obtained the 1,759 bp fragment between the *Mlu* 1 and *Xho* 1 sites covering the 5′ promoter region of mouse *PLD1* by PCR amplification using forward and reverse primes: 5′-GGGACGCGT-CCCTCTCCTTCCCCTGAGGT-3′, and reverse primer; 5′-GGG-CTCGAGTGGGGCCCTGGCAAAAA-3′, respectively. The *Mlu* 1 and *Xho* 1 enzyme sites were underlined and double underlined, respectively. After confirming the sequence context, the 1,759 bp *PLD1* promoter fragment was inserted into the *Mlu* 1 and *Xho* 1 sites of the pGL3 basic vector (Promega, Madison, WI) (−1,759 bp/Luc). To obtain −26 bp/Luc reporter construct, −1,759 bp/Luc promoter construct was digested by *Mlu* 1 and *Eco*R 1. Then, the product was blunted by Blunting high kit (Toyobo, Osaka, Japan), and was ligated with Ligation high kit (Toyobo). Similarly, −1,239 bp/Luc, −896 bp/Luc, −570 bp/Luc, −431 bp/Luc, −338 bp/Luc, −314 bp WT (containing wild-type C/EBP motifs)/Luc, −314 bp M1 (containing mutated distal C/EBP motif)/Luc, −314 bp M2 (containing mutated proximal C/EBP motif)/Luc, −314 bp MD (containing two mutated C/

EBP motifs)/Luc, and −231 bp/Luc promoter constructs were obtained by PCR amplification and ligation using the primer sets described below. The reverse primer was GL primer 2. Forward primers:

−1,239 bp/Luc: 5′-GGGACGCGTAAGGCTGAGTGAACTGTTTAGG-G-3′,
−896 bp/Luc: 5′-GGGACGCGTTGTGTTCATGTGGAGGCCAGAG-3′,
−570 bp/Luc: 5′-GGGACGCGTATGGAAAGCTAGCGACAGTATGA-3′,
−431 bp/Luc: 5′-TTTACGCGTTGTACTGCCCTTGTTTAACGTG-3′,
−338 bp/Luc: 5′-TTTACGCGTTGTTTTAAGTGTGTGCTCTGGC-3′,
−314 bp WT/Luc: 5′-TTTACGCGTTTCTTCGGCAACCAGTGCA-A-3′,
−314 bp M1/Luc: 5′-TTTACGCGTTTCaaCGGCcACCAGTGC-3′,
−314 bp M2/Luc: 5′-TTTACGCGTTTCTTCGGCAACCAGaatAAAA-CTG-3′,
−314 bp MD/Luc: 5′-TTTACGCGTTTCaaCGGCcACCAGaatAAAA-CT-3′,
−231 bp/Luc: 5′-TTTACGCGTTATGGGGCGTGGTTTCAAGT-3′.

The *Mlu* 1 site was underlined. Mutated C/EBP motifs were shown as lowercase letters.

TRANSFECTION OF *C/EBPα* AND *C/EBPβ* EXPRESSION VECTORS TO NIH3T3 CELLS

Five micrograms of each of mock-pCDNA3.1 (+), *C/EBPα*, and *C/EBPβ* expression vector was transfected to NIH3T3 cells using Lipofectin Reagent (Invitrogen). After 36 h incubation, Western blotting sample and total RNA from respective cells were obtained.

PROMOTER ANALYSIS

HEK293 cells were transfected with 2 μg of promoter plasmid containing the various lengths of *PLD1* Luc reporter and 1 μg of *β-gal* expression vector using Lipofectin (Invitrogen). After 2 days, luciferase and β-gal activities were measured. Promoter activity was normalized with β-gal activity as described before [Kikuchi et al., 2007].

WESTERN BLOTTING

Western blotting using anti-PLD1, anti-PLD2, and anti-β-actin antibodies was performed as described previously [Ohguchi et al., 2004].

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extract was prepared from 3T3-L1 cells treated with or without IBMX or IDX for 36 h. EMSA was performed according to the method described previously [Kikuchi et al., 2007]. The sequences of wild-type and mutated oligos used were as follows:

Wild-type probe: GCTTTCTTCGGCAACCAGTGCAAAACTGAAG.
C/EBP motif double mutated probe: GCTTTCaaCGGCcACCAGaat-AAAACTGAAG.

Mutated C/EBP motifs are described in lowercase letters. Supershift assay was performed using anti-C/EBPα and anti-C/EBPβ antibodies (Santa Cruz Biotechnology, Inc.).

CHROMATIN IMMUNOPRECIPITATION ASSAY (ChIP)

ChIP assay was performed as described previously [Kikuchi et al., 2007]. For immunoprecipitation, normal rabbit (control) IgG, anti-C/EBPα, or anti-C/EBPβ antibody (1 μg/sample) were added and incubated overnight at 4°C. After DNA extraction, the *PLD1* 5′ promoter region containing C/EBP motif was amplified by PCR using the following primers: 5′-ATCAGCACCCAACTTAGGCAACA-3′ (forward); and 5′-CGGTCAAGCCCACAGAGCA-3′ (reverse). The size of the PCR product was 309 bp.

STATISTICAL ANALYSIS

Statistical significance was analyzed by Student's *t*-test using Statview ver. 5 (SAS Institute, Inc., Cary, NC).

RESULTS

The effects of the respective inducers were examined for differentiation of 3T3-L1 cells. Using Oil-Red-O staining, we examined 3T3-L1 cells treated with various combinations of adipocyte differentiation inducers. Among the three inducers (Ins, Dex, and IBMX), no single reagent was effective in generating the lipid droplet. However, the combination of IBMX with DEX or Ins was effective in generating the lipid droplet, although the degree of lipid accumulation was much less compared with the three-inducer cocktail, IDX (data not shown). These observations were compatible with those in previous report [Caprio et al., 2007].

Figure 1 demonstrates the rapid increase of PLD1 but not PLD2 protein by IDX. PLD1 began to increase 24 h after IDX treatment, whereas PLD2 level remained at almost the same level through the differentiation process. We also examined several key transcription factors known to be involved in adipocyte differentiation. Expression of C/EBPβ and C/EBPδ, but not C/EBPα and PPARγ

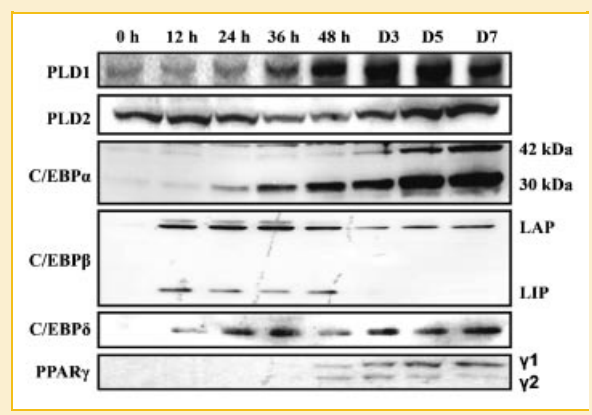

Fig. 1. Western blotting of PLD isozymes and adipocyte differentiation-related transcription factors. 3T3-L1 cells were induced to adipocyte lineage with IDX. Samples for Western blotting were taken at indicated times. PLD1, PLD2, C/EBPα, C/EBPβ, C/EBPδ, and PPARγ were analyzed using antibodies as described in the Materials and Methods Section. Liver activator protein (LAP) and liver inhibitory protein (LIP) denote two fragments of C/EBPβ, whereas γ1 and γ2 show two components of PPARγ. Experiments were repeated several times with similar results.

(γ1 and γ2), preceded the PLD1 expression. Among the two components of C/EBPβ, the expression level of liver inhibitory protein (LIP) was enhanced from 12 to 48 h and then declined, whereas liver activator protein (LAP) maintained an increased level after 12 h with IDX.

The *PLD1* mRNA level changed in parallel to its protein level. After 24 h with IDX, *PLD1* but not *PLD2* mRNA level began to increase (Fig. 2A). Our Oil-Red-O staining suggested that maintenance of the cAMP level by IBMX (0.5 μM) is important for lipid droplet accumulation. We further examined the effect of various reagents that can increase cellular cAMP level. Figure 2B (at the ratio of PLD/β-actin) showed that IBMX, an inhibitor of phosphodiesterase, forskolin (10 μM), an activator of adenylate cyclase, and 10^{-3} M of dbcAMP could induce *PLD1* mRNA compared with control.

As the next step, we performed *PLD1* promoter analysis using HEK293 cells, because these cells have been used as an effective replacement of 3T3-L1 cells with low transfection efficiency [Scheepers et al., 2001]. Figure 3A shows that the region responsible for IDX was located between −570 bp and the first exon. Furthermore, it shows that IBMX as a single reagent induced *PLD1* promoter activity significantly compared with other two inducers, DEX and Ins (Fig. 3B). Previous analysis of adipocyte differentiation using 3T3-L1 cells have shown that the sequential activation of transcription factors (C/EBPβ, C/EBPα, and PAPRγ) is sufficient to induce the whole differentiation process of 3T3-L1 cells [Rosen et al., 2000]. Figure 3C illustrates the effect of IDX and IBMX on C/EBPβ protein level, showing that IDX and IBMX induced C/EBPβ and PLD1, respectively. On the other hand, C/EBPα was not affected (data not shown).

Figure 4A,B demonstrates the results of overexpression of C/EBPβ or C/EBPα in NIH3T3 cells. NIH3T3 cells were used for its high transfection efficiency. C/EBPβ but not C/EBPα induced PLD1 protein and *PLD1* mRNA. *PLD2* expression was not affected by overexpressing C/EBPβ and C/EBPα. C/EBPβ also increased *PLD1* promoter activity in HEK293 cells (Fig. 4C).

Furthermore, truncation and mutation of the promoter region revealed that two C/EBP motifs located between −314 and −231 bp were the regions responsible for IBMX stimulation (Fig. 5A,B). These two C/EBP motifs (shown as the distal site of Fig. 5B, left) were located very close, and both were needed for full responsiveness of IBMX. These results strongly suggest that the pathway starting from increased cellular cAMP by IBMX induced C/EBPβ expression followed by *PLD1* mRNA increase by the transcription factor, C/EBPβ, during the early phase of adipocyte differentiation.

EMSA was performed to confirm the direct binding of C/EBPβ and *PLD1* 5′ promoter. An oligoprobe containing the two C/EBP motifs described above produced a single shifted band, while the mutated probe produced a faint band (Fig. 6A). The cold competitor abolished this band, suggesting the specificity of this band formation. Anti-C/EBPβ, but not anti-C/EBPα, antibody reduced the shifted band formation considerably.

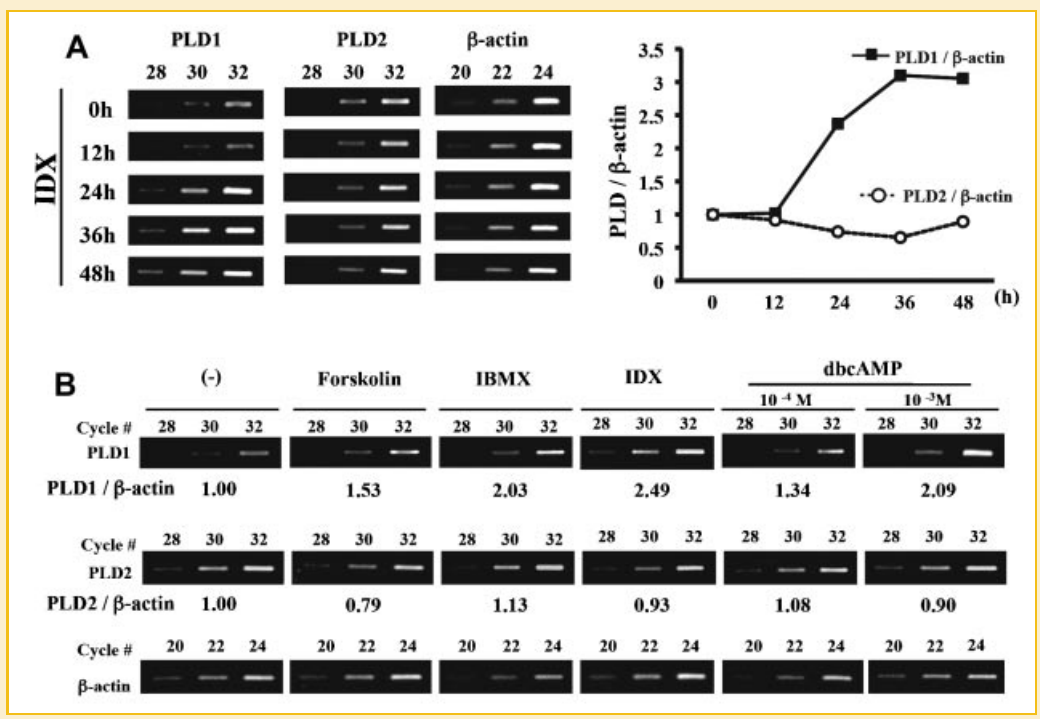

Fig. 2. Semi-quantitative RT-PCR of *PLD1* and *PLD2*. A: Left part: During the treatment of 3T3-L1 cells with IDX over 48 h, RNA samples were collected at indicated times, and semi-quantitative RT-PCR was performed according to the procedures as described in the Materials and Methods Section. The right part indicates the relative mRNA levels calculated from (A, left). The data at 0 h were regarded as 1.0, respectively. B: 3T3-L1 cells were treated for 36 h with or without IDX, IBMX, forskolin (10 μM), and dbcAMP. *PLD1*, *PLD2*, and *β-actin* mRNAs were measured by the same semi-quantitative RT-PCR method. The relative mRNA levels of *PLD1* and *PLD2* (PLD/β-actin ratio) of control cells were determined as 1.0.

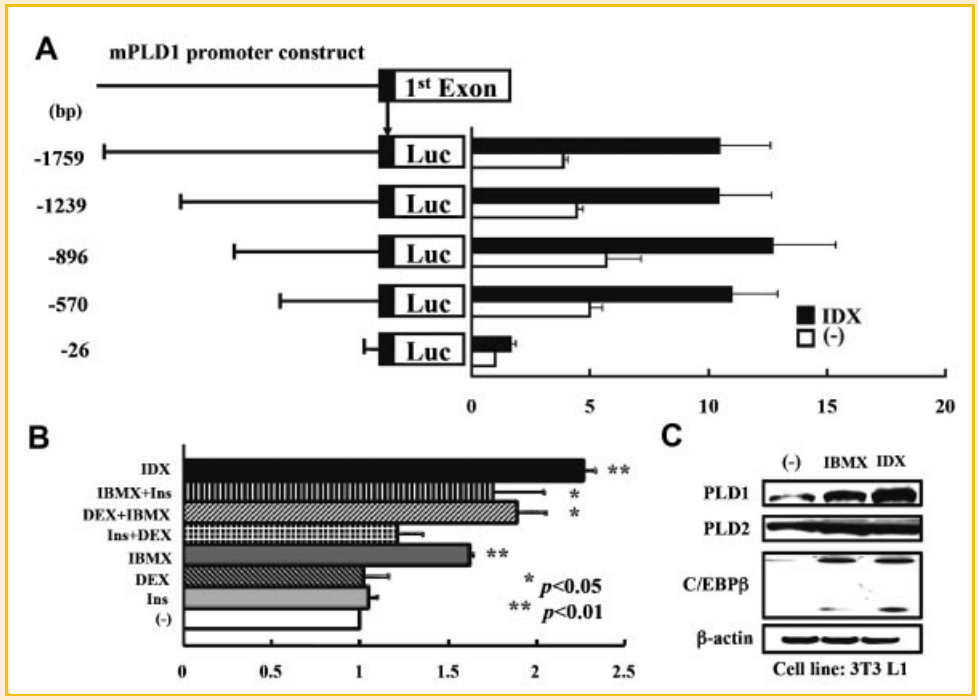

Fig. 3. The effects of IDX and IBMX on PLD expression and *PLD1* promoter activity. A,B: HEK293 cells were cultured and were transfected with reporters containing various lengths of *PLD1* 5′ promoter region and *β-gal* expression vector using Lipofectin (Invitrogen). Four hours after transfection, the medium was replaced with 10% FCS in DMEM. An inducer cocktail, IDX, various combinations of two inducers, or a single inducer were added to the cells. After 48 h, cells were collected and the relative reporter activity was measured according to the Materials and Methods Section. In (A), the data of −26 bp/Luc without IDX were regarded as 1.0. Results are expressed as the mean ± SD. Experiments were repeated at least three times. In experiment (B), −570 bp/Luc vector was used. The data of −570 bp Luc without any inducer were regarded as 1.0. C: 3T3-L1 cells were treated with IDX or IBMX for 48 h. Western blotting was performed using anti-PLD1, anti-PLD2, anti-C/EBPα, anti-C/EBPβ, and anti-β-actin antibody, respectively.

ChIP assay showed that anti-C/EBPβ, but not anti-C/EBPα, antibody immunoprecipitated the *PLD1* 5′ promoter region containing two C/EBP motifs, but only when cells were treated with either IBMX or with IDX (Fig. 6B), supporting our reporter assay and EMSA.

DISCUSSION

The presence of PLD activity in adipocyte tissue has been previously reported [Millar et al., 1999]. Among two PLD isoforms, PLD1 is known to be involved in oleic acid-induced adipocyte differentiation of NIH3T3 cells [Nakamura et al., 2005; Andersson et al., 2006]. Exogenous PA or activated PLD was shown to stimulate lipid droplet formation [Marchesan et al., 2003]. In the present study, we analyzed endogenous gene expression of *PLD* isoforms during adipocyte differentiation of 3T3-L1 cells and showed that *PLD1*, but not *PLD2*, mRNA increased during the early period of adipocyte differentiation by the inducer cocktail, IDX. Several groups have reported their microarray data of the adipocyte differentiation model of 3T3-L1 cells [Guo and Liao, 2000; Burton et al., 2004]. However, *PLD1* has not yet been described.

Interestingly, Oil-Red-O staining revealed that IBMX in combination with either Ins or DEX induced mild lipid droplet formation, whereas Ins + DEX did not (data not shown). A similar finding has been recently reported [Petersen et al., 2008]. Increased *PLD1* mRNA is thought to be mediated by increased cellular cAMP, because IBMX, an inhibitor of cAMP phosphoesterase inhibitor, or an agonist of adenylate cyclase, forskolin, and a high concentration of cell permeable cAMP, dbcAMP, increased *PLD1* mRNA (Fig. 2). Actually, although cAMP-induced *PLD1* mRNA expression has been reported in rat PC12 cells and mouse C6 glioma cells [Yoshimura et al., 1996; Hayakawa et al., 1999], the regulatory mechanism has not been elucidated. Protein kinase A (PKA) has been believed to be the major protein activated by cAMP; however, the involvement of the cAMP/PKA pathways in adipocyte differentiation of the 3T3-L1 cells is controversial [Kato et al., 2007; Li et al., 2008; Martini et al., 2009]. A PKA-independent pathway has also been proposed in adipocyte differentiation [Petersen et al., 2008]. Further analysis is necessary to evaluate the involvement of PKA in this increased *PLD1* gene expression by cAMP.

It has been reported that PLD is involved in lipid droplet formation [Marchesan et al., 2003; Nakamura et al., 2005; Andersson et al., 2006]. However, in our preliminary experiments, 1-butanol, a specific PLD inhibitor did not inhibit lipid droplet accumulation completely (data not shown), suggesting that PLD expression is not a sole factor to induce adipocyte differentiation. We further analyzed the expression of several genes known as the target of PLD [Kato et al., 2005; Park et al., 2009]. Among them, *MMP-9* and its inhibitor, *TIMP-1* mRNAs of cells treated with IDX

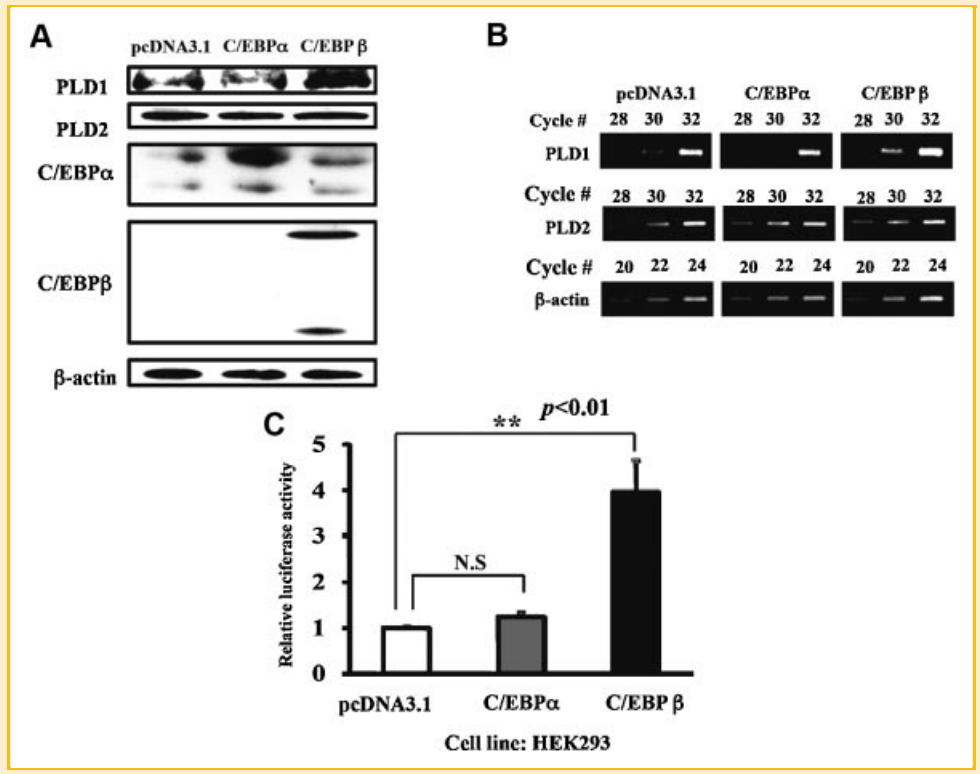

Fig. 4. Effects of C/EBP overexpression on PLD protein, *PLD* mRNA, and *PLD1* promoter activity. A,B: Mock (pcDNA3.1), C/EBPα, and C/EBPβ expression vectors were used for transfection of HEK293 cells. Thirty-six hours after transfection, samples for Western blotting (A) and the semi-quantitative RT-PCR (B) were collected. Western blotting and semi-quantitative RT-PCR were performed as described in the Materials and Methods Section. C: HEK293 cells were cultured and transfected with mock-pcDNA3.1 vector, C/EBPα, or C/EBPβ expression vector together with −338 bp *PLD1*/Luc and *β-gal* expression vector which are shown in Figure 5A. Luciferase activity was measured 48 h after transfection. The relative promoter activity was calculated as Luc/β-gal as shown in Figure 3. Experiments were repeated three times with similar results.

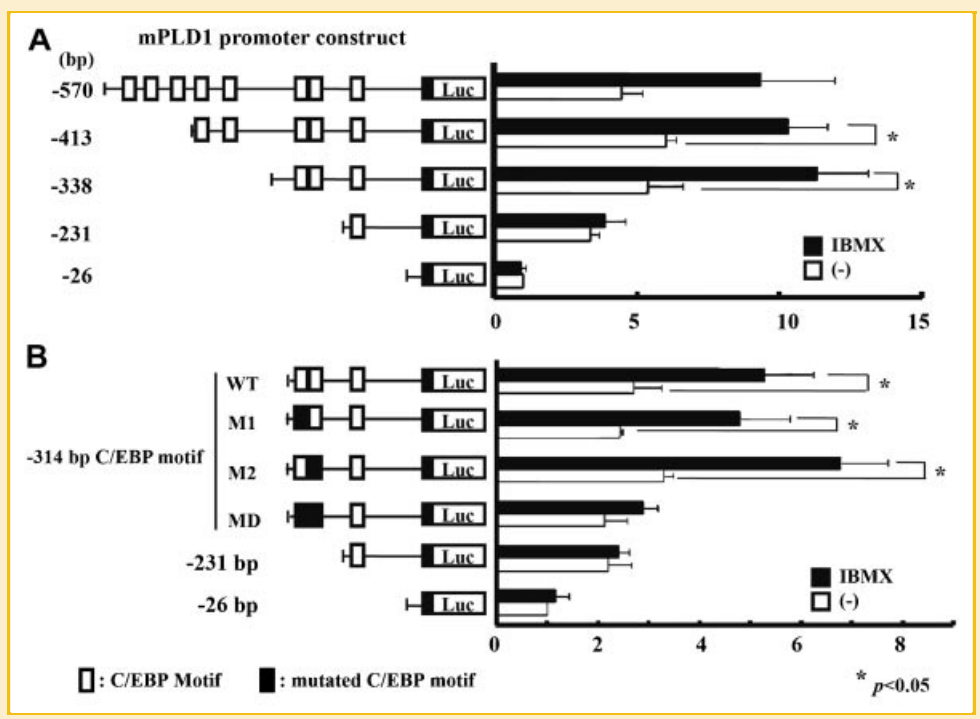

Fig. 5. Identification of IBMX responsive elements of the *PLD1* 5′-promoter. A,B: Various truncated reporters as well as those with mutated C/EBP motifs were prepared as described in the Materials and Methods Section. Transfection of vectors and reporter assays were performed as described in the Materials and Methods Section. At the right part, the solid and open bars denote IBMX treatment and no treatment, respectively. On the left, the putative C/EBP motifs (open boxes) and its mutation (solid box) are shown. The −26 bp/Luc without IBMX treatment was regarded as 1.0.

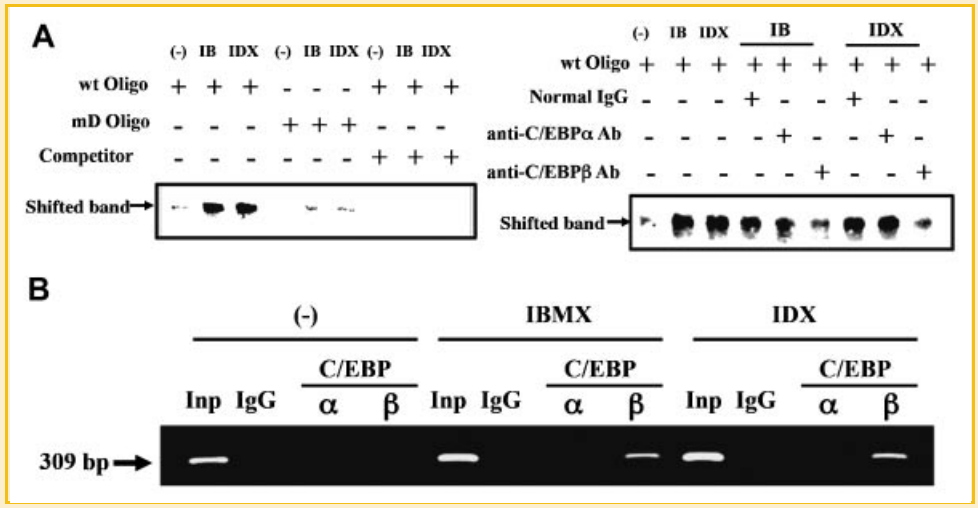

Fig. 6. EMSA and ChIP assays. EMSA and ChIP assays were performed according to the procedure described in the Materials and Methods Section. A: Thirty-six hours after IBMX or IDX treatment of 3T3-L1 cells, nuclear extract was prepared and incubated with either labeled wild-type or mutated oligoprobe. A control (−) sample was prepared from cells without adipocyte induction. In some experiments, non-labeled oligo was added to the reaction mixture as the cold competitor. For the supershift experiments, each nuclear extract was preincubated with either normal rabbit IgG (Santa Cruz Biotechnology, Inc.), anti-C/EBPα (Santa Cruz Biotechnology, Inc.), or C/EBPβ antibody (Santa Cruz Biotechnology, Inc.) before mixing with the labeled probe. B: ChIP assay was performed using control 3T3-L1, IBMX-treated 3T3-L1, and IDX-treated 3T3-L1 cells. Cells were crosslinked with formaldehyde as described in the Materials and Methods Section. For immunoprecipitation, normal rabbit IgG, anti-C/EBPα, and anti-C/EBPβ antibody was added and incubated for 24 h at 4°C. After crosslinking was reversed with heating, DNA was extracted and PCR was performed with the primer set described in the Materials and Methods Section. The expected PCR product was 309 bp in length.

were inhibited by 1-butanol, but not its counterpart, *t*-butanol (data not shown), suggesting PLD1 plays a role in matrix alternation during adipocyte differentiation. Moreover, expression of sphingosine kinase 1 is reported to be induced in 3T3-L1 cells during the adipocyte differentiation process, indicating an involvement in differentiation [Hashimoto et al., 2009]. PA, a product of PLD, is known to activate and to translocate SPHK1 to cell membrane [Olivera et al., 1996; Johnson et al., 2002; Delon et al., 2004], suggesting the possible implication of PLD in the SPHK1 activation. In this context, further analysis is needed to evaluate the role of PLD1 in the adipocyte differentiation.

The regulatory mechanism of adipocyte differentiation has been explained as the consequence of the sequential activation of transcription factors starting from C/EBPβ and followed by C/EBPα/peroxisome proliferators-activated receptor (PRAR) [Rosen et al., 2000]. Sequential changes in the expression level of PLD1 and C/EBP proteins lead us to provide the hypothesis that the increases of *PLD1* mRNA and PLD1 protein were due to increased C/EBPβ. Overexpression experiments using C/EBPβ and C/EBPα expression vectors support this hypothesis. Similarly, the stimulation of C/EBP homologous protein (CHOP) expression and transcriptional activity by cAMP has been reported [Pomerance et al., 2003]. Our promoter analysis revealed that the region of 5′ promoter of *PLD1* (−338 and −231 bp from the first exon) contained two putative C/EBP motifs, and direct binding of C/EBPβ to these C/EBP motifs was shown by both the EMSA and ChIP assays (Fig. 6).

Taken together, our current analysis shows for the first time that PLD1 expression increases during the early phase of adipocyte differentiation of 3T3-L1 cells and elucidates the regulatory mechanism of *PLD1* gene expression by increased cellular cAMP via the direct action of increased C/EBPβ transcription factor during IDX-induced differentiation. The relevance of this early increase of *PLD1* gene expression toward the following late phase of adipocyte differentiation is an interesting issue to be explored by future analysis.

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