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Transcriptional activation of Cidec by PPARγ2 in adipocyte

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ABSTRACT

Cidec is a lipid droplet-associated protein, which inhibits lipolysis, leading to the accumulation of triglycerides in adipocytes. However, the transcriptional regulation of Cidec in adipocyte remains unknown. In the present study we investigated that the mouse Cidec transcript is regulated by PPARγ2. After the differentiation of adipocyte, the expression pattern of Cidec was similar to that of PPARγ2. In the presence of a PPARγ agonist, the level of Cidec mRNA was highly increased. In addition, putative PPRE sites were identified in the Cidec promoter. By chromatin immunoprecipitation assay and reporter assay, we observed the binding of PPARγ2 to the promoter of Cidec. Gel shift assay and the mutagenesis study were showed that the −219/−207 region of the Cidec promoter could function as a PPRE of the Cidec promoter. These results suggest that PPARγ2 is required for the transcriptional activity of Cidec during adipogenesis, which could be contributed to understand the molecular mechanism of lipid droplet formation in adipocytes.

The adipose tissue regulates energy homeostasis, which stores excess energy in the form of triglycerides and releases in case of necessity. Adipokines are secreted in adipose tissue for metabolic regulation [1]. The abnormal regulation of the energy metabolism leads to metabolic disorders such as diabetes, hypertension, and hyperlipidemia.

PPARγ, one of nuclear receptors, is a master regulator for adipocyte differentiation [2–4]. PPARγ also regulates lipid metabolism, insulin sensitivity, cardiovascular disease, inflammation, organ development and tumor formation [5,6]. PPARγ has two isoforms, PPARγ1 and PPARγ2, which are created by alternative splicing. PPARγ1 is expressed in several tissues including the liver, whereas PPARγ2 is exclusively expressed in adipose tissue [7]. PPARγ2 activates target genes by binding to promoter regions after forming a complex with retinoid X receptor-α (RXRα). The binding sites of the target genes, which consist of a direct repeat of the consensus half-site motif (AGGTCA) spaced by a single nucleotide, are referred to as peroxisome proliferator response elements (PPREs) [8].

In eukaryotic cells, excess lipids are stored in lipid droplets. The more lipids accumulate in cells, the more lipid droplets are fused into large size. Lipid droplets are enclosed by a single layer of phospholipids and associated a number of proteins [9–11]. Recent studies report that these proteins participate in the regulation of lipid storage, the inhibition of lipolysis and the motility of lipid droplet [12,13]. Cidec is identified as a novel lipid droplet-associated protein, which plays role to suppress lipolysis, accumulate triglyceride and stimulate the size of lipid droplet [14,15].

In hepatic steatosis, the expression of Cidec is induced by PPARγ1, although Cidec is not expressed in normal liver [16,17]. Inflammatory cytokine TNF-α down-regulates its level [18]. However the regulatory mechanism of Cidec during adipogenesis is still unclear. Therefore, in the present study we demonstrated that the transcriptional activation of Cidec is regulated by PPARγ2 in adipocyte.

Materials and methods

Plasmid constructs. Firefly luciferase reporter pGL3 vector was constructed to contain 1.5 kbp (pGL3-Cidec 1.5 kb), 994 bp (pGL3-Cidec 994 bp), 170 bp (pGL3-Cidec 170 bp), and 94 bp (pGL3-Cidec 94 bp) from the translation start site of the mouse Cidec gene (GenBank Accession No. NC_000072). These fragments were generated by PCR using the specific primers listed in Table 1. The PCR products were digested with the restriction enzymes KpnI and XhoI to generate appropriate protruding ends. Site-directed mutagenesis of the luciferase promoter (pGL3-Cidec 994 bp) was performed using the QuikChange site-directed mutagenesis kit following the instructions provided by the supplier. The PPRE2 sequence TGCC*CT*CTTGC*CT* in the Cidec promoter was changed to TGCC*AG*CTTGC*AG*. Successful mutagenesis was confirmed by sequence analysis.

Cell culture. 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% calf serum. At two days after reaching confluency (designed as day 0), the differentiation of preadipocytes was initiated by a solution (1 μg/ml insulin, 1 μM DEX, and 0.5 mM MIX in

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Table 1
Sequences of oligonucleotides.

Gene	Usage	D[a]	Sequences (5′–3′)
Cidec	RT-PCR, real-time PCR	F	AGC TAG CCC TTT CCC AGA AG
		R	CCT TGT AGC AGT GCA GGT CA
	ChIP (PPRE1/PPRE2)	F	GCG GAA CTC AGA CCA TAA GC
		R	GAA AAG AGC ATG GGG AAT GA
	ChIP (PPRE3)	F	TTT GGT GGC ACA CAC CTT TA
		R	GGG GTT GGA GAT CTG TCT CA
	ChIP (PPRE4)	F	AAC AGT CAG CTT GCC TCT GC
		R	GGC CAC ACA GTG AAA GGT CT
	pGL3-Cidec1.5 kb	F	GGT ACC TTA AAC ACA AAA CAG TCA GC
	pGL3-Cidec994 bp	F	GGT ACC GAG ACT TTA TAG CTC AGG CT
	pGL3-Cidec170 bp	F	GGT ACC ACA TGC CTT CTT CCC CGT T
	pGL3-Cidec94 bp	F	GGT ACC ATT CCC CAT GCT CTT TTC
	pGL3-5′UTR	R	CTC GAG CTC CTG GCA ATA CCG CGT G
PPARγ2g2	RT-PCR	F	CCC TGG CAA AGC ATT TGT AT
		R	GAA ACT GGC ACC CTT GAA AA
	Real-time PCR	F	CCC TGG CAA AGC ATT TGT AT
		R	TTG ATT TTA TCT TCT CCC ATC ATT

[a] Direction-F, forward; R, reverse.

DMEM supplemented with 10% FBS). After 48 h, the culture medium was replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. Cells were fed every other day.

Reverse transcriptase PCR and real-time PCR. Total RNA from 3T3-L1 cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The synthesis of cDNA was carried out using MMLV-reverse transcriptase (Takara, Shinga, Japan) and oligo-dT primers (Invitrogen, Carlsbad, CA, USA). The cDNA was subjected to PCR amplification using Maxime PCR PreMix (iNtRON Biotechnology Inc., Seoul, Korea) and gene-specific primers (Table 1). PCR was performed under the following conditions: 2 min at 95 °C; 27 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C; and 5 min at 72 °C. PCR products were separated by electrophoresis through a 1.5% agarose gel. Real-time quantitations were performed using the Bio-Rad iCycler iQ system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The fluorescence threshold value was calculated using the iCycle iQ system software. The reverse transcription reaction mixture was incubated with specific primers (Table 1) using iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA). Data were processed by the comparative cycle threshold method and expressed as fold increase relative to the basal transcription level. The amount of target mRNA was normalized by determining the level of GAPDH mRNA.

Chromatin immunoprecipitation (ChIP) assays. Differentiated (day 6) 3T3-L1 adipocytes were fixed in 18.5% formaldehyde for 10 min at room temperature and neutralized with 125 mmol/l glycine for 5 min. After washing with PBS, cell lysates were sonicated to produce chromatin fragments averaging 500 bp in size. Fragmented chromatins were added into the ChIP dilution buffer [16.7 mM Tris–HCl (pH8.1), 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, and inhibitor cocktail]. Samples were incubated with anti-PPARγ antibody (Abcam, Cambridge, UK) at 4 °C. Immune complexes were precipitated with Protein A resin (Millipore Upstate, Lake Placid, NY, USA) and were transferred to mini columns (Bio-Rad, Hercules, CA, USA) for washing. Finally the beads were eluted using TE buffer. DNA–protein cross-links were reversed by incubation with 10% Chelex at 95 °C for 10 min. The DNA was treated with proteinase K (Roche, Indianapolis, IN, USA) at 55 °C for 30 min. Precipitated chromatins were used as the template for PCR. PCR was performed using the following conditions: 5 min at 95 °C followed by 36 cycles of 30 s at 95 °C, 30 s at 61 °C, and 30 s at 72 °C. PCR was carried out using primer pairs described in Table 1.

Transient transfection and luciferase reporter assays. The reporter plasmids were transfected into preadipocytes or mature adipocytes (day 4) cells using the Fugene 6 reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. At 48 h post-transfection luciferase activities were measured with the Dual-Luciferase assay kit (Promega, Madison, WI, USA). In addition, 3T3-L1 preadipocytes were co-transfected with reporter plasmids in combination with both pSG5 myc-mPPARγ2 and pCMV-mRXRα. The cells were incubated with culture medium containing 50 μM GW9662. After transfection, cells were harvested at 24 h. Luciferase activity was measured using a GloMax20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from Cos-7 cells transfected with both pSG5 myc-mPPARγ2 and pCMV-mRXRα. For EMSA, a LightShift Chemiluminescent EMSA Kit (Pierce Biotech, Rockford, IL, USA) was used according to the manufacturer's instructions. The ^{32}P-labeled PPRE (DR-1, 25 mM) was incubated with 2 μg nuclear extract in 20 μl binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT) containing 2.5 mg/ml BSA, 20 pg/μl poly (dI-dC) and 1 mM DTT. The binding shift was challenged with a 5-fold of double stranded Cidec-PPRE1, Cidec-PPRE2 or the mutant forms of both PPRE1 and PPRE2. Binding affinities to the candidate PPREs were assessed by the efficiencies of competition with a probe containing the conserved PPRE (DR-1). Double-stranded oligonucleotides were generated by annealing complementary nucleotides. PPRE (DR-1) probes were labeled by [α-^{32}P]dCTP using a Klenow DNA polymerase. The competition experiments were performed by adding a 5-fold excess of unlabeled probe to the reaction mixture. The following oligonucleotides were used: PPRE (DR-1 conserved oligonucleotide) 5′-TCTGAGAGGTCAAAGGTCATCCCCT-3′; PPRE1(Cidec promoter region −113/−101) 5′-ATGCAAATCCCTAGGGGAAAGTTTCGAAC TCAT-3′; PPRE2(Cidec promoter region −219/−207) 5′-ATGC TGC TTCTGTGCCCTCTTGCCTAGTGCCAT-3′; PPRE1-mu (mutated PPRE1): 5′-ATGCAAATCCCTCTGGGAACTTTTCGAACTCAT-3′; PPRE2-mu (mutated PPRE2) 5′-ATGCTGCTTCTGTGCCAGCTTGCAGAGTGCC AT-3′. Electrophoresis was performed in a 0.5 x Tris-borate buffer at room temperature at 180 V for 90 min on 5% polyacrylamide gels. The gels were then dried and protein-DNA complex formation was analyzed by autoradiography.

Results

The expression of Cidec during adipocyte differentiation

To identify the potential regulatory actions of Cidec, we determined its expression pattern in 3T3-L1 cells during adipogenesis. In preadipocyte, the expression of Cidec was not detected, whereas the signal of Cidec transcripts was dramatically elevated in mature 3T3-L1 cells (Fig. 1A). Interestingly, these expression patterns were similar to those of PPARγ2. To quantify these observations we conducted real-time PCR analysis. The levels of Cidec mRNA in mature adipocytes were highly increased more than 15,000-fold in comparison to those in preadipocytes (Fig. 1B). The expression level of PPARγ2 mRNA also increased significantly 36-fold at day 6 of differentiation compared to that in preadipocytes. To investigate the transcriptional regulation of Cidec by PPARγ2, thiazolidinedione (TZD) PPARγ agonist was treated into adipocyte cells. As a result, the expression of Cidec was strongly increased in cells depending on TZD treatments (Fig. 1C). These results suggest that the transcription of Cidec may be regulated by PPARγ during adipocyte differentiation.

Determination of the putative PPAR binding site in the Cidec promoter

In order to analyze the putative PPRE sites of the Cidec promoter, the MatInspector program was used [19]. As results, four putative

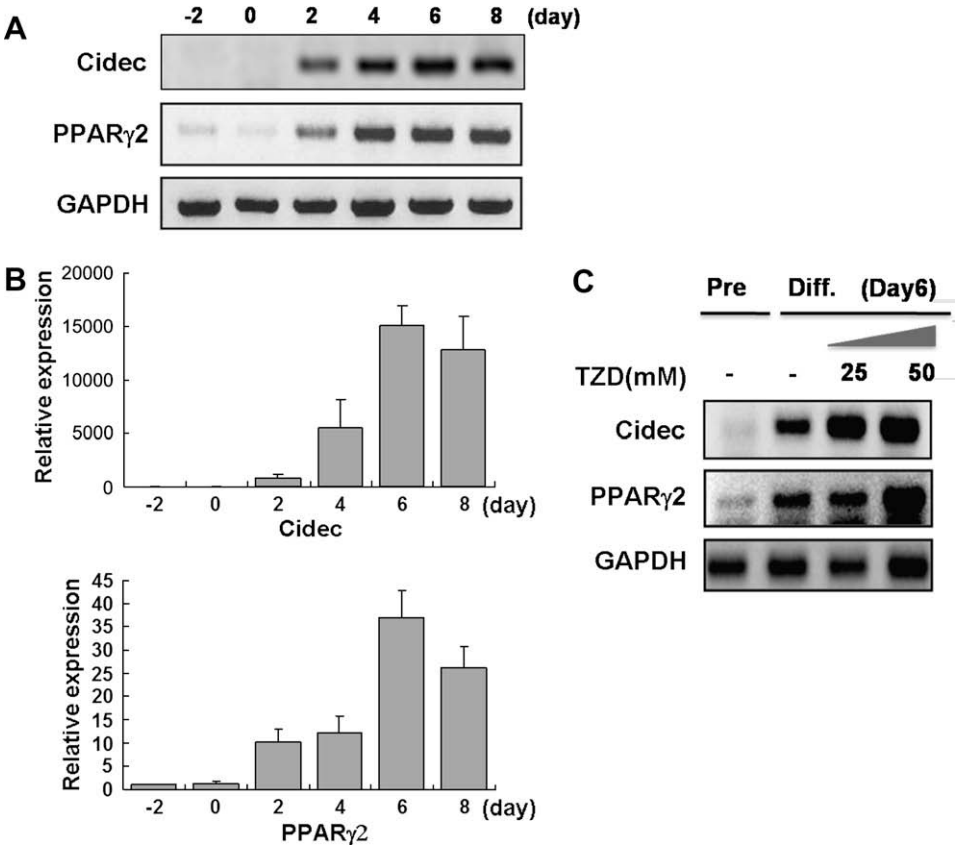

Fig. 1. The expression of Cidec and PPARγ2 in 3T3-L1 cells during adipogenesis. (A) The levels of Cidec and PPARγ2 mRNA were determined by RT-PCR. Cytoplasmic RNA was isolated from differentiating 3T3-L1 cells and assayed by RT-PCR. (B) The levels of Cidec and PPARγ2 mRNAs were quantified by real-time PCR. Quantities of mRNA were normalized with that of GAPDH mRNA. The data are presented as means ± S.E. (C) Effect of PPARγ agonist TZD on the expression of Cidec mRNA. Differentiated (day 6) 3T3-L1 cells were treated with TZD and assayed by RT-PCR.

PPAR binding sites were identified in the 2000 bp from the transcription start site of Cidec (Fig. 2A): −1808 to −1796 (denoted as PPRE4), −1294 to −1282 (denoted as PPRE3), −219 to −207 (denoted as PPRE2), and −113 to −101 (denoted as PPRE1). In order to determine whether PPARγ2 is recruited into these PPRE sites of the Cidec promoter, we conducted ChIP assay. As shown in Fig. 2B, we observed PCR products corresponding to PPRE1/PPRE2 and to PPRE3, but not to PPRE4. These results demonstrate that PPARγ2 binds to the PPRE1/2 and PPRE3 regions of the Cidec promoter.

The transcriptional activation of Cidec by PPARγ2

Our next goal was to identify which of these PPARγ2 binding sites functions to regulate the transcriptional activity of Cidec. Several PPRE deleted mutants were constructed for luciferase reporter assays (Fig. 3A). Since PPRE4 exhibited no binding affinity for PPARγ2 in ChIP assays, we escaped it. In preadipocyte, the promoter activity was not measured in cells transfected with any reporter vectors. However, in mature adipocyte, we observed the enhanced luciferase activity in cells transfected with the pGL3-Cidec 994 bp and the pGL3-Cidec 1.5 kb constructs containing PPRE1/PPRE2 domains. The pGL3-Cidec 170 bp vector containing only PPRE1 did not show any activity (Fig. 3B). Next, both PPARγ2 and RXRα plasmids were co-transfected into preadipocyte to determine the PPARγ2-dependency of the Cidec promoter activity. In the absence of PPARγ2/RXRα heterodimer, the luciferase activity in both pGL3-Cidec 994 bp and pGL3-Cidec 1.5 kb vectors was not detected, but cells expressing exogenous PPARγ2/RXRα protein showed the increased reporter activity (Fig. 3C). In addition, the up-regulated activity of the Cidec promoter was significantly decreased in cells treated with PPAR antagonist GW9662 (Fig. 3C). Taken together, these results suggest that PPARγ2 regulates the transcriptional activation of Cidec by binding to the PPRE2 region.

The PPARγ2-induced transcriptional activation of Cidec via PPRE2 region

Based on our previous experiments, the functional binding site of Cidec for PPAR γ2 is PPRE2 region (−219/−207). Sequence alignment reveals that the PPRE2 is highly conserved. To confirm the active PPARγ2 binding site of the Cidec promoter, we conducted site-directed mutagenesis by modifying the conserved sites of PPRE2 (Fig. 4A). As shown in Fig. 4B, the activity of PPRE2 mutant was completely eliminated, whereas intact PPRE exhibited high promoter activity. In order to determine that the PPARγ2/RXRα heterodimer binds to the PPRE region of Cidec promoter, an electrophoretic mobility shift assay was conducted with the PPRE (DR-1) as a probe. In absence of competitors, both PPARγ2/RXRα heterodimer (lane 3) was bound to the probe. However, when 5-fold excess competitors were added to the reaction mixture, PPRE2 competed with the binding of the PPARγ2/RXRα heterodimer (lane 5), whereas PPRE1 and mutant competitors, P1mu and P2mu) did not compete with the heterodimer (lane 4, 6, and 7). These results indicate that the PPRE2 region (−219/−207) of the Cidec promoter could function for the transcriptional activation induced by PPARγ2.

Discussion

PPARγ plays a role in lipid metabolism. The PPARγ agonist thiazolidinedione reduces plasma free fatty acids and increases lipid

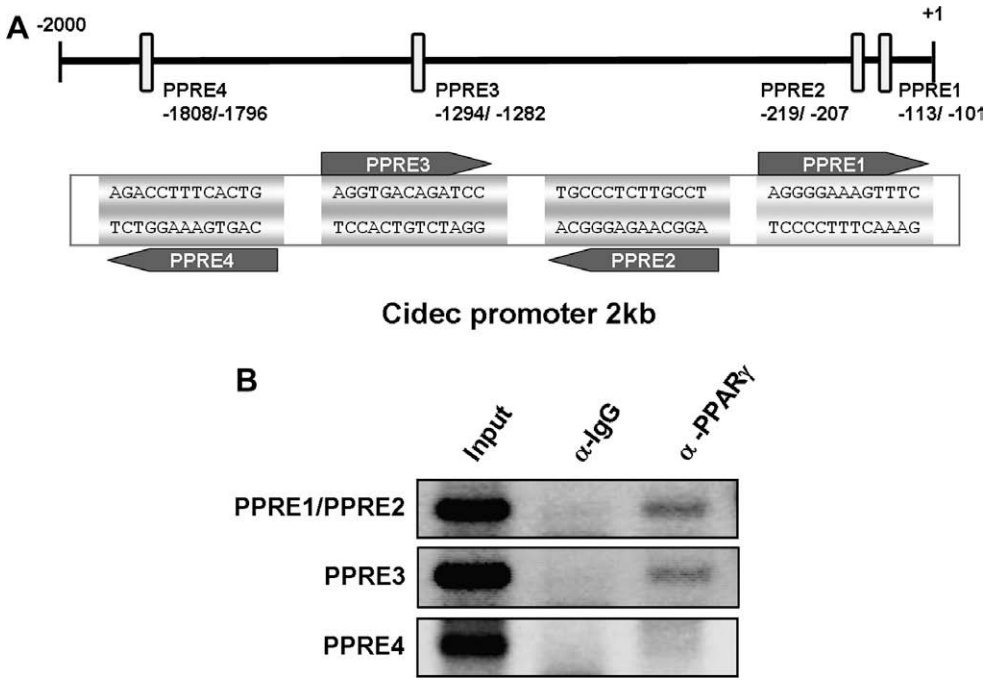

Fig. 2. Association of PPARγ2 with the putative PPRE regions in mouse Cidec promoter. (A) Location of the putative PPARγ-binding sites is shown in −2 kb of the Cidec promoter. The schematic structure and sequence of four putative PPREs were indicated. Arrows show the strand containing PPRE. (B) The binding of PPARγ2 to PPRE regions of the Cidec promoter was analyzed by ChIP assays. After immunoprecipitation, PPRE regions were amplified by PCR reaction. Total chromatins were indicated as 'input'. Pre-immune IgG was used as a negative control.

uptake in adipose tissue *in vivo* [20]. These physiological reactions are induced by which the activated PPARγ regulates the transcriptional activation of genes in association with fatty acids uptake, lipogenesis, triglyceride synthesis and lipolysis [21]. Lipid droplets consist of triglycerides enclosed phospholipids, which involve on lipid homeostasis by the regulation of lipid metabolism as well as lipid storage [22]. One of lipid droplet-associated proteins, perilipin, regulates the storage of triglycerides [23]. Perilipin is highly expressed in the differentiated adipocyte and its transcription is controlled by PPARγ2 on the −1986/−1974 PPRE region of perilipin promoter [24]. Cidec colocalizes with perilipin on the surface of lipid droplets in adipocyte [14]. Cells depleted Cidec mRNA increase lipolysis and show the

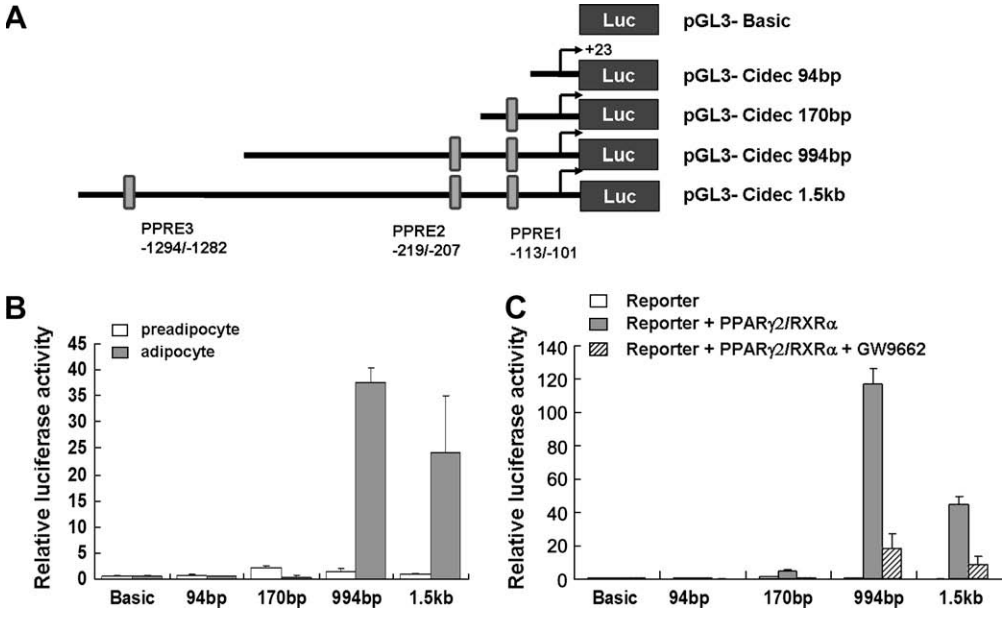

Fig. 3. Identification of the functional PPRE region in the Cidec promoter. (A) Schematic structure of various PPRE deleted mutants in the Cidec promoter. The PPRE deleted mutants for Cidec promoter were designed as pGL3-Cidec 94 bp (−94/+23), pGL3-Cidec 170 bp (−170/+23), pGL3-Cidec 994 bp (−994/+23) and pGL3-Cidec 1.5 kb (−1500/+23). The pGL3-Basic vector is used as a control. (B) Truncated mutants were transfected into preadipocytes and mature adipocytes (day 4). After 48 h, luciferase reporter activity was measured. (C) 3T3-L1 preadipocyte cells were co-transfected with or without both PPARγ2 and RXRα vectors. GW9662 (50 μM) was applied to cells as a specific PPARγ antagonist. After 24 h, luciferase reporter activity was assayed. These experiments were conducted as independent experiments in triplicate. The data are presented as means ± S.E.

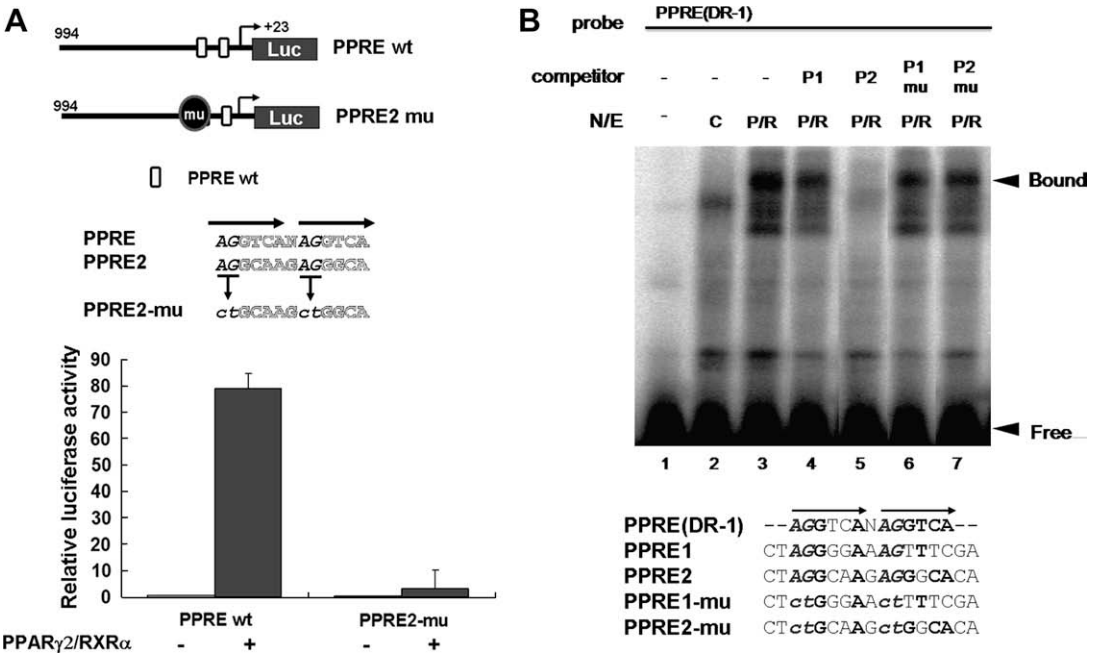

Fig. 4. The PPARγ2-dependent transcriptional activation of the Cidec gene through the PPRE2 region. (A) The sequences are shown in alignment with a consensus PPRE. The conserved PPRE2 domain of Cidec promoter was mutated as indicated. (B) Luciferase reporter vectors were co-transfected into 3T3-L1 preadipocytes with or without the PPARγ2/RXRα expression vectors. Reporter activity was measured 48 h later. These studies were conducted as independent experiments in triplicate. The data are presented as means ± SE. (B) Competitive EMSA was performed to determine specific binding of PPARγ2/RXRα heterodimer to the Cidec promoter. PPRE1 (P1), PPRE2 (P2), PPRE1 mutant (P1mu) and PPRE2 mutant (P2mu) were used as competitors. N/E, nuclear extracts; C, control; P/R, PPARγ2/RXRα.

fragmented small lipid droplets by inhibiting the fusion of lipid droplets in mature adipocytes [14,15]. In the present study, we showed that PPARγ 2 regulates the transcriptional activation of Cidec. Therefore, based on previous studies and our data, we insist that PPARγ2 as a nuclear receptor could participate in the overall process for the formation and enlargement of lipid droplets.

Cidec belongs to CIDE (cell death-inducing DNA fragmentation factor-like effector) family including Cidea and Cideb. CIDEs were originally identified proteins with high homology to proapoptotic DNA fragmentation factor DFF 45 [25]. However, recent study reveals that CIDEs involve in the regulation of energy metabolism. In comparison with other Cide proteins, Cidea is well studied, which is highly expressed in brown adipose tissues and regulates the energy expenditure [26–28]. Transcriptional activation of Cidea is known to be regulated by Sp1/Sp3 and PPARα/PPARγ [29,30]. However, Cidec is still unclear how to regulate its transcription in adipocytes. It is reported that the expression of Cidec is decreased in cells treated with TNF-α and is upregulated in hepatic steatosis inducing the overexpression of PPARγ1 [16–18]. In the present study, we show that the transcriptional activity of Cidec is induced by PPARγ2 in differentiated adipocytes. Therefore, we believe that PPARγ2 regulates energy homeostasis by the transcriptional regulation of CIDEs including Cidea and Cidec. These findings may have important implications concerning the molecular basic mechanisms involved in adipocyte differentiation and lipid metabolism.

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