**Prolactin regulatory element binding protein involved in cAMP-mediated suppression of adiponectin gene**

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Prolactin regulatory element binding protein involved in cAMP-mediated suppression of adiponectin gene

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ABSTRACT

OBJECTIVES: Adiponectin (ApN) has several protective effects against diabetes and atherosclerosis. However, the detailed mechanisms of the regulation of the ApN gene have not yet been clarified. PREB (prolactin regulatory element binding) protein has been identified as a factor that regulates insulin gene expression in the pancreas. PREB is located not only in the pancreas but also in adipose tissue; however, its role in adipose tissue is not known. METHODS: To analyze the effects of PREB on adiponectin gene transcription, we employed a reporter gene assay and electrophoretic mobility shift assay (EMSA). In the cells expressing or knocking down the PREB, adiponectin expression was determined. RESULTS: PREB was located mainly in the nuclei of adipose tissue and its cell line, 3T3-L1 cells. The nuclear extract contained adiponectin promoter binding activity that was super-shifted by PREB antiserum in EMSA studies. In the 3T3-L1 cells, the co-expression of PREB and the adiponectin promoter inhibited the activity of the latter. The addition of cAMP to the cells increased PREB expression in a dose-dependent manner. A deletional analysis of the adiponectin promoter showed that the PREB-responsive cis-element in the adiponectin promoter mediated the transcriptional effect of PREB, while a mutant of this motif in the adiponectin promoter abrogated the effect of PREB, as well as that of cAMP. Furthermore, cells expressing or knocking down PREB exhibited decreased and increased adiponectin expression, respectively. CONCLUSIONS: These results demonstrate that PREB may contribute to the regulation of adiponectin gene transcription, in response to cAMP activation in adipocytes.

Key words: adiponectin, adipose tissue, cAMP, PREB, transcription
INTRODUCTION

Adiponectin (ApN) is an adipose-specific secretory protein that acts as an anti-diabetic and anti-atherosclerotic molecule [1-3]. Furthermore, a number of clinical trials have shown that subjects with high levels of circulating adiponectin tend to be protected against type 2 diabetes and myocardial infarction. Previous study reported that higher concentrations of adiponectin protected against later development of type 2 diabetes (incidence rate ratio 0.63; 95% confidence interval (CI) 0.43–0.92), p=0.02) [4]. Pischon et al. reported that participants in the highest compared with the lowest quintile of adiponectin levels had a significantly decreased risk of myocardial infarction (relative risk, 0.39; 95% CI, 0.23-0.64; P<0.001) [5]. Several factors regulate adiponectin gene expression, including other adipocytokines such as tumor necrosis factor-α and IL-6 [1, 6]; transcription factors such as peroxisome proliferator-activated receptor-γ and liver receptor homolog-1 [7]; CCAAT/enhancer binding protein (C/EBP); nuclear transcription factor-Y (NF-Y); and sterol regulatory element-binding protein-1c [8]. A previous report showed that cAMP inhibited ApN gene expression in human visceral adipose tissue. Likewise, cAMP down-regulated ApN mRNAs in cultured mouse explants from visceral and subcutaneous regions [9]. The amount of ApN released into the medium decreased concomitantly; however, the underlying mechanism is not clear.

The prolactin regulatory element binding (PREB) gene encodes a 1.9-kb mRNA that translates into a transcription factor that binds to and activates the basal prolactin promoter activity. The PREB protein also mediates the protein kinase A
(PKA) action in the pituitary gland [10, 11]. The primary sequence of the PREB protein contains two potential trans-regulatory PQ-rich domains and three regions of high similarity with the WD-repeat, thus making it a member of a eukaryotic family of WD-repeat proteins. Members of this ever-growing family are involved in multiple cellular functions that include signal transduction, RNA processing, cytoskeletal assembly, and vesicle trafficking [12]. The PREB has similarities to a subset of this family that acts as gene regulators.

Although PREB transcripts are present in the adipose tissue—in addition to pituitary, heart, skeletal muscle, and pancreas [11]—its role in the adipose tissue is not known. PREB participates in the PKA stimulation of prolactin promoter activity, suggesting a role for this protein in cAMP-mediated responses [10]. Since PREB is present in the adipose tissue and is known to mediate the actions of cAMP, we wondered whether PREB might participate in controlling the ApN gene expression by cAMP. Therefore, we examined the effect of PREB on the transcription of the ApN gene. Our findings show that the PREB binds to the ApN promoter and also regulates the ApN secretion in response to cAMP. These results suggest that the PREB is an important transcriptional factor that regulates the ApN gene in adipose tissue.

MATERIAL AND METHODS

Cell culture

The 3T3-L1 cells originated from a mouse adipose cell line. The
undifferentiated 3T3-L1 cells were cultured in Dulbecco’s Modified Eagle’s Medium with 25 mM glucose (DMEM, Gibco-BRL, Tokyo, Japan) and supplemented with 10% newborn bovine serum (FBS; Thermo Electron Co., Melbourne, Australia), 4 mM L-Glutamine, 1 mM Sodium Pyruvate, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. To introduce differentiation, 3T3-L1 cells were grown in DMEM supplemented with 10% FBS, and the standard differentiation inducing mix: insulin (100 nM, Sigma, Saint Louis,), dexamethasone (0.25 mM, Sigma), and isobutylmethylxanthine (IBMX, 0.5 mM, Sigma), for the first two days. For the next two days, the medium was supplemented only with 100 nM insulin. From day 5, the cells were switched back to plain DMEM with 10% FBS. The cells would be fully differentiated on day 10 and could be used until days 15–17. COS-7 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% FBS. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

In vitro transcription and translation

The pcDNA3.1 (+) vector carrying PREB cDNA or solely vector was transcribed in vitro with T7 RNA polymerase (Gibco-BRL), as previously described [13]. The RNA product was translated with a rabbit reticulocyte lysate system (Promega, San Luis Obispo, CA).

Immunohistochemical localization of PREB

Freshly isolated adipose tissues were fixed with phosphate-buffered
formalin (pH 7.0) overnight, then paraffin wax embedded and subsequently deparaffinized. Sections of 5 µm each were incubated overnight with a monoclonal anti-PREB antibody, as described previously [14]. After washing in phosphate buffered saline (PBS), slides were incubated with biotinylated goat anti-rat IgG at 5 µg/ml (Vector Laboratories) for 1 h at room temperature. Slides were developed using a peroxidase detection kit (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA).

Polymerase chain reaction (PCR)

Total RNA was extracted from the mouse adipose tissue, as well as undifferentiated and differentiated 3T3-L1 cells. The primer sequences for mouse PREB mRNA were: sense primer 5'-ATGATCGTGATGGTGCCGTC-3' and antisense primer 5'-ACTGAACCTGCAGGTGCTGA-3'. The sequences were amplified and analyzed under identical conditions, using previously described primers [13]. β-actin served as the control.

Electrophoretic mobility shift analysis (EMSA)

Proteins of PREB and the control were synthesized by using the TNT Quick Coupled Transcription/Translation Systems (Promega Corporation, Madison, WI). Three synthetic DNA duplexes span binding site A1 (5'-GAAAGAGTGGGAGTATCATGATGGTACATTAGTG-3'), binding site A2 (5'-GACCCCTGAACCATCATTCTGAGG-3') and binding site A3
(5'-CACTCAGAAACATGCTGAATTATTTGCTTACC-3'). The corresponding sequences of three mutant probes show probe A1 (5'-CACTAATTGTCACAGAGGACTCCCACTCTTTT-3'), probe A2 (5'-CCTCAAGTAAAAGAGGGTGTTCAGGGGTC-3') and probe A3 (5'-GGTAAGGACAGAGCCCCGCTGGAAATTTCGAGT-3'). The three mutant probes and 1P (-66 TGCCTGATTATATATATATTCATGAAGGTGTCGAA-32) (Nihon Bioservice, Asagiri, Japan) used in these studies were radio-labeled at the 5'-end by incubating each strand separately with [$\gamma^{32}$P]-ATP and polynucleotic kinase prior to annealing, as described previously [15]. In competition analysis, a 50-fold and 200-fold molar excess of unlabeled competitor 1P was added to the reaction prior to the addition of the nuclear extracts.

**Western blot analysis**

Cells were washed, scraped into PBS, and lysed, as described previously [15]. The proteins were resuspended under reducing conditions and 15 µg was fractionated by size on 7.5% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. The membranes were incubated for 1 h at 4°C with 0.1% Tween 20 in PBS (PBS-T) containing anti-PREB antiserum (diluted 1:2,000) as described previously [13]. The antibody binding was visualized using a chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).
Transfection of 3T3-L1 cells and luciferase reporter gene assay

To confirm the transcriptional regulation of the adiponectin promoter by PREB, we used a plasmid construct containing the mouse adiponectin promoter obtained by PCR amplification, cloned in front of the luciferase reporter gene as previously described [8]. Wild type (-984 WT LUC) contains the mouse adiponectin gene sequences spanning the region from -984 to +1, linked to the luciferase reporter gene [8]. The constructs, mutant types -984 mA1-LUC, -984 mA2-LUC, and -984 mA3-LUC lack the consensus sequence A1, A2, and A3 respectively, and were generated as described previously [16]. Site-directed mutagenesis within the first 984 bp of the mouse adiponectin promoter gene was performed according to the manufacturer’s instructions (Stratagene, La Jolla, CA); in brief, the PREB core sequence of consensus motif was disrupted by altering four base pairs (5’-ATCA to 5’-CCTC) in the A1 element derived from the parent construct -984 WT-LUC. Purified reporter plasmid was transfected into 3T3-L1 (at 80% confluence) using a conventional cationic liposome transfection method (Lipofectamine; Life Technologies, Gaithersburg, MD).

Generation of adenovirus and adenovirus treatment

The full-length rat PREB cDNA was inserted into the pShuttle vector plasmid, as previously described [10]. Adenovirus expressing the PREB (Ad-PREB) was constructed according to the instructions for the Adeno-X Expression System kit (CLONTECH laboratories, Inc., Palo Alto, CA). As a control, Adeno-X-lacZ
adenovirus (Ad-LacZ) was generated. Adenoviruses were amplified in HEK 293 cells, and purified and concentrated to $10^{12}$ plaque-forming units per ml (pfu/ml) by CsCl ultracentrifugation. Expression of PREB was transduced by incubation with the Ad-PREB adenoviruses for three hours at a multiplicity of infection of 1,000 pfu/cells.

**Transfection of siRNA**

The siRNAs were designed to target the following cDNA sequences: scrambled, 5'-CCGTTCTGTACAGGGAGTACT-3'; and PREB siRNA, 5'-AATGGCGTGCACTTTCTGCAG-3' [13]. Transfection of PREB siRNA was performed using siPORT Lipid (Ambion, Austin, TX, USA) as described previously [16]. Adiponectin protein expression was examined using Western blot analysis, three days after transfection.

**Experimental animals**

Eight-week-old male C57BKS/J db/db mice (db/db) and their age matched control lean mice (lean) were purchased from The Jackson Laboratory (Bar harbor, ME). Protocol employed in this experiment was reviewed and approved by the Kagawa University Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent diet until 4 months of age. After overnight fasting, the mice were killed and the epididymal fat tissues collected and stored at –80°C for nuclear protein isolation.
Statistical analysis

Statistical comparisons were made by one-way analysis of variance and Student’s \( t \)-test, with \( P < 0.05 \) considered significant.

RESULTS

Presence of the PREB transcript in the mouse adipose tissue and its cell line, 3T3-L1 cells

We examined the tissue distribution of PREB by RT-PCR analysis (Fig.1A). Results showed that PREB was expressed in various mouse tissues. We examined PREB mRNA in mouse adipose tissue, the adipose cell line, undifferentiated and differentiated 3T3-L1, and INS-1 cells using RT-PCR analyses. Results showed the presence of PREB mRNA of the predicted size [10] in mouse adipose tissue, undifferentiated and differentiated 3T3-L1, and INS-1 (Fig.1B).

In addition, we performed immunohistochemical analyses using anti-PREB antibody, to examine the sub-cellular localization of PREB in human adipose tissue. Figure 1C shows the immunostaining of PREB mainly located in the nuclei of adipose tissue.

To further extend our previous finding that PREB is located in the nuclei of cells [10], we isolated nuclear extract from undifferentiated and differentiated 3T3-L1 cells. The Western blot analysis showed the presence of a major protein band of approximately 45-kD that matched the expected size of PREB protein in
undifferentiated and differentiated 3T3-L1 cells, especially in undifferentiated nuclear extract (Fig. 1D). Together, these findings show that PREB was mainly located in nuclei of adipose tissue and 3T3-L1 cells.

**PREB suppressed the adiponectin promoter activity and protein expression in COS-7 cells**

To determine whether PREB affected the transcriptional activity of the adiponectin promoter, we used the -984WT LUC construct comprised a full-length mouse adiponectin promoter co-transfected into COS-7 cells with a PREB expression vector, because a Northern blot analysis of this cell line yielded undetectable levels of PREB [13]. PREB repressed adiponectin promoter activity by 40% (p<0.05) in COS-7 cells (Fig. 2A). To examine whether PREB affected adiponectin protein expression, we measured the total adiponectin protein levels in the cells infected with the Ad-PREB adenovirus. As shown in Fig. 2B, the cells infected with the Ad-PREB adenovirus decreased adiponectin protein expression in the cells; in contrast, the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was not affected (Fig. 2B). Although in vitro experiments on cultured cells, as mentioned above, have helped to elucidate the role of PREB on adiponection gene, it remains unknown whether PREB has the same effect on an in vivo model. To answer this question, we used db/db mice, an animal model of diabetes mellitus, and the lean nondiabetic counter parts, lean mice. Since adiponectin expression is significantly reduced in the fat tissue of diabetic animal models [17, 18, 19], we examined the involvement of
PREB in the repression of adiponectin expression in db/db mice. Plasma adiponectin levels in db/db mice (13 ± 3.2 ng/ml) were significantly lower than those in lean mice (22 ± 2.5 ng/ml) as similar as a previous report. Figure 2C showed that the abundance of the PREB protein increased in the fat tissue of db/db mice.

cAMP stimulates accumulation of PREB protein in 3T3-L1 cells

Therefore, we examined the effect of this metabolic regulator on PREB expression in both undifferentiated and differentiated 3T3-L1 cells. Western blots probed with a PREB-specific antiserum showed that the relative abundance of the PREB protein increased in response to cAMP in a dose-dependent manner (Fig. 3); in contrast, the basal level of GAPDH was not affected by cAMP. Furthermore, the relative abundance of the PREB mRNA also increased following treatment with cAMP in a dose-dependent manner (data not shown). These results suggest that cAMP stimulates the expression of PREB in both undifferentiated and differentiated 3T3-L1 cells.

PREB interacts with A1 cis-acting element of adiponectin promoter

Next, we searched for a DNA motif within the adiponectin promoter that may bind PREB. An examination of the promoter sequence revealed a 7-nucleotide-motif (ATTGTCC) corresponding to the deduced PREB core-binding-element (PCBE) of the prolactin gene [10]. A DNA homology search revealed the presence of three putative PREB binding motifs within the mouse
adiponectin promoter region: elements A1 (-398 to -395), A2 (-324 to -321), and A3 (-285 to -273). We generated three mutant adiponectin promoters mutA1 LUC (A1 deletion), mutA2 LUC (A2 deletion), and mutA3 LUC (A3 deletion) derived from the parent construct -984 WT LUC and co-transfected them into the 3T3-L1 cells, along with a PREB-expressing plasmid and measured the transcriptional activity (Fig. 4). Results in Figure 4 show that the presence of PREB reduced wild type adiponectin promoter activity by 60% (p<0.05); this reduction was dependent on the amount of PREB. The results showed that PREB had no effect on A1-mutated adiponectin promoter activity, but it still had effects on the A2 and A3 mutants (Fig. 4). These findings suggest that the A1 element is required for the actions of PREB on adiponectin promoter activity.

To determine whether the binding activity to the A1 motif was indeed due to the PREB, we synthesized the PREB protein using in vitro transcription/translation. The product arising from this approach yielded a major single band with a mass of 45-kDa, as determined using Western blot analysis (data not shown). Whether the in vitro synthesized PREB could bind the radio-labeled A1 motif was studied using EMSA. Results showed that PREB expressed using in vitro transcription/translation bound to A1 element (Fig. 5A). PREB binds directly to site 1P of the prolactin promoter [10]. We used an EMSA competition analysis of PREB binding to the 1P site in the presence of a 50- or 200-fold molar excess of unlabeled A1 element competitor DNA. Results (Fig. 5B) show that the nuclear extract from the 3T3-L1 cells were bound to 1P. The addition of excess A1 element DNA competed for
PREB binding to the radio-labeled 1P probe. To confirm that PREB bound to the A1 element directory, we employed the super shift assay with a specific antiserum against PREB. Our results (Fig. 3B, 3C) show that the protein-DNA complex was shifted by the addition of anti-PREB antiserum but not by pre-immune serum.

**PREB knock-down or site-directed mutagenesis of the A1 abrogates the effect of cAMP.**

Next, we tested whether the PREB might affect the cAMP-suppressed adiponectin protein expression. To address this issue, 3T3-L1 cells were treated with a specific or scrambled PREB siRNA and then exposed to a fixed amount (10^{-5} M) of cAMP. Our results showed that the adiponectin protein levels decreased in the cells treated with the scrambled siRNA and cAMP (Fig. 6A), but the adiponectin protein expression was not decreased in the cells treated with the PREB-specific siRNA and cAMP (Fig. 6A). To further show that the role of PREB is important in cAMP-suppressed adiponectin promoter activity, we used the reporter gene constructs with/without the mutation, within the PREB binding motif. As shown, cAMP treatment inhibited the transcriptional activity of the A2 and A3 mutants of the adiponectin promoter (data not shown), but not that of the mutant A1 of the adiponectin promoter (Fig. 6B).

**DISCUSSION**

PREB cDNA was recently isolated from a rat pituitary cDNA library, and
the protein product was shown to transactivate prolactin promoter activity [10]. PREB mRNA transcripts were present not only in the pituitary, but a strong signal was also present in both the pancreas and the adipose tissue. Recently, we reported that PREB might participate in the regulation of insulin gene transcription and insulin secretion, in response to glucose stimulation [13]. In this report, we examined the role of PREB in the regulation of adiponectin expression in mouse adipocytes and their cell line, 3T3-L1; we observed that the over-expression of PREB decreased adiponectin protein expression in adipocytes (Fig. 2). These findings are consistent with the idea that PREB is a negative transcriptional factor for adiponectin.

Adiponectin forms oligomers and seems to circulate in the plasma as a homotrimer or as larger complexes of 12–15 subunits. One of its most interesting features—as observed in rodents, monkeys, and humans—is that its expression in adipose tissue and plasma is lower in obese and diabetic subjects [4, 20]. Adiponectin increases the insulin sensitivity associated with the activation of insulin signaling and glucose uptake [21]; furthermore, the administration of full-length adiponectin lowers plasma glucose levels by suppressing hepatic glucose production in obese and diabetic mice [3].

cAMP accelerated the spontaneous decline of the adiponectin mRNAs. Its effect was unrelated to enhance lipolysis, but was substantially reversed by actinomycin. cAMP could destabilize the messengers or inhibit the transcription of the ApN gene, via direct or indirect mechanisms. If cAMP were only to destabilize ApN mRNAs directly, one would expect no change in the “blunted” levels of the
mRNA when cAMP and actinomycin D were combined. As this was not the case, other mechanisms must be involved. cAMP could directly inhibit ApN gene transcription. No cAMP-response element (CRE) has been identified in the promoter of the human or mouse ApN gene, but this does not rule out the possibility that the transcription factors activated by cAMP may bind to non-conventional sites [22]. Eventually, cAMP could act indirectly, possibly through the enhanced transcription of the aforementioned inhibitory protein. We have indicated that cAMP stimulated the expression of PREB in 3T3-L1 cells. In the PREB promoter, there are many consensus-binding motifs for transcriptional factors including CRE (K. Murao and H. Imachi, unpublished results). It may respond to cAMP stimulation to increase the transcription of PREB. Further studies will be needed to determine the detailed mechanisms involved in the regulation of the PREB gene.

An analysis of the primary sequence of PREB reveals that it is a novel transcriptional factor that is distinct from Pit-1. The PREB protein has three motifs—WD I, WD II, and WD III—with significant degrees of homology to the consensus WD repeat; this suggests that PREB is a member of the WD-repeat protein superfamily [12]. The highly conserved WD-repeats within PREB demonstrate a sequence similarity to a subset of proteins belonging to this family, which consists of proteins that are gene regulators [12]. Unlike other WD-repeat proteins, PREB exerts transcriptional regulation, as it has been shown to stimulate gene expression by directly binding to DNA [10, 13]. Our results show that PREB binds to the adiponectin promoter and inhibits adiponectin promoter activity in 3T3L1 cells.
Our results show that PREB binds to the adiponectin promoter, while a mutant PREB binding site abrogates the effect of not only PREB but also cAMP. These results suggest an alternative mechanism in which PREB may be involved in the cAMP-mediated inhibition of adiponectin promoter activity. A previous report indicated that PREB could mediate the PKA-induced stimulation of prolactin promoter activity [10], thus suggesting a role for this protein in cAMP-mediated transcriptional responses. A possible model for this potential role of PREB may involve the activation of PREB via PKA-mediated phosphorylation. Although it is not yet known whether PREB can serve as a PKA substrate either in vitro or in vivo, the predicted sequence of this protein contains a number of motifs resembling consensus PKA phosphorylation sites [23]. Further studies will be needed, to determine the specific regulatory mechanisms that enable PREB to regulate adiponectin gene transcription.

A recent report indicates that a transcriptional factor activating enhancer-binding protein-2β (AP-2β) inhibits the transcriptional activity of the adiponectin gene through its binding in 3T3-L1 adipocytes [24]. In this study, we have reported that the newly transcriptional factor PREB was a negative regulator of the adiponectin gene in adipocytes. Based on these findings, we hypothesize that the PREB transcriptional factor is one of the candidate genes causing dysfunction in adipocytes, leading to atherosclerosis and type 2 diabetes. Recent reports indicated that increased adiponectin levels associated with increased expression of heme oxygenase (HO)-1 in obese rats and obese diabetic mice [18, 19]. Further studies will
be necessary to determine the role of PREB on HO-adiponectin system in these animals.

In summary, our findings show that PREB can function as a transcriptional regulator of the adiponectin promoter and thus mediate the effect of cAMP. PREB binds to the adiponectin promoter, and in cells expressing PREB, it decreases adiponectin expression. Further investigations will help us define a possible physiological role for PREB in adipose tissue.
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FIGURE LEGENDS

Figure 1. Expression of PREB.  A) Tissue distribution of PREB in various mouse tissues.  RT-PCR analysis of PREB transcripts in mouse tissues.  β-actin was used as a control for equal RNA loading.  Lane1; maker, lane 2; adipose tissue, lane 3; liver, lane 4; kidney, lane 5; pancreas, lane 6; spleen.  B) The presence of PREB mRNA in mouse adipose tissue, undifferentiated and differentiated 3T3-L1 cells, and INS-1 cells was examined by RT-PCR analysis of 3 µg of poly A RNA from these cells; β-actin was used as a control for equal RNA loading.  Lane 1, marker; lane 2, water as a negative control; lane 3, INS-1; lane 4, undifferentiated 3T3-L1; lane 5, differentiated 3T3-L1; lane 6, adipose tissue.  C) Immunohistochemical localization of PREB in human adipose tissue.  Immunoperoxidase staining of PREB was presented in the nuclear of human adipose tissue (II), and the tissue stained with control serum was negative (I).  D) PREB protein expression.  Nuclear extract from differentiated 3T3-L1 cells was subjected to Western blot analysis.  Abundance of β-Tubulin served as a control and was found at the bottom of each lane.  Lane 1, undifferentiated 3T3-L1 nuclear extract; lane 2, differentiated 3T3-L1 nuclear extract.  The ratio of adiponectin to β-Tubulin is shown as a percentage of control.  Each data point shows the mean ± SE (n=3) of separate experiments.  The asterisk denotes a significant difference (P<0.01).

Figure 2. The role of PREB in the regulation of adiponectin expression in COS-7 cells.  A) Dishes (60 mm) of COS-7 cells were co-transfected with 1 µg of
-984WT-LUC plus the indicated amounts of PREB expression vector, then incubated for 24 h prior to cell harvest. Total amount of DNA per well was equalized by the addition of empty vector for PREB expression vector. All assays were corrected for β-galactosidase activity and equal total amounts of protein per reaction were employed. The results were expressed as luciferase activity relative to control cells, arbitrarily set at 100. Each data point shows the mean ± SE (n=3) of separate transfections. The asterisk denotes a significant difference (P<0.01). B) PREB gene and control (LacZ) was transfected into undifferentiated 3T3-L1 cells by adenovirus. After 48 h, the expression of adiponectin was examined using Western blot analysis. The ratio of adiponectin to GAPDH is shown as a percentage of control. Each data point shows the mean ± SE (n=3) of separate experiments. The asterisk denotes a significant difference (P<0.01). C) Increased level of PREB in db/db mice. PREB in nuclear protein from adipose tissue was detected by western blot analysis probed with an anti-PREB antibody. Abundance of TFIID served as the control and is shown at the bottom of each lane. The PREB/TFIID ratio is shown as % of the control in the figure. Each data point shows the mean ± SEM of 5 experiments for each group. The asterisk denotes a significant difference (P<0.01).

L: lean mice, db/db: db/db mice.

**Figure 3. Effects of cAMP on PREB protein accumulation in 3T3-L1 cells.**

Protein extract of differentiated 3T3-L1 cells treated with varying concentrations of cAMP for 24 h. Western blot analysis was performed to examine PREB
accumulation (upper panel). Analysis of GAPDH as a control is shown at the bottom of each lane. The ratio of PREB to GAPDH is shown as the percentage of control (lower panel). Each data point shows the mean ± SE (n = 3) of separate experiments. The asterisk denotes a significant difference ($P < 0.05$).

**Figure 4. Site directed mutagenesis of the A1 abrogates the response to PREB.**

The upper panel shows a schematic diagram of the adiponectin promoter constructs. The reporter genes containing -984 WT LUC, -984 A1 mutant LUC (-984 mA1 LUC), -984 A2 mutant LUC (-984 mA2 LUC), and -984 A3 mutant LUC (-984 mA3 LUC) were constructed. Cells were transfected with the mutated reporter genes plus the indicated amounts of PREB expression vector, and promoter activity was determined by luciferase assay. All assays were corrected for β-galactosidase activity and total amounts of protein per reaction were identical. The results are expressed as luciferase activities relative to that of control cells arbitrarily set at 100. Each data point shows the mean ± SE (n=3) of separate transfections ($P<0.05$).

**Figure 5. PREB exhibits DNA sequence-specific binding to adiponectin promoter element A1.** EMSA was carried out using the A1 oligonucleotide as a probe and synthesized PREB and control protein by in vitro transcription/translation. Panel A shows an autoradiograph of an EMSA of synthesized control and PREB proteins bound to radio-labeled probe A1. Lane 1, probe A1 only; lane 2, synthetic PREB protein; lane 3, synthetic protein as control. Panel B shows competition
reactions of binding pattern of synthetic protein PREB, with radio-labeled probe A1 and an indicated excess amount of cold 1P. Lane 1, probe only; lane 2, no competitor; lane 3, 50-fold M excess of unlabeled 1P; lane 4, 200-fold M excess of unlabeled 1P. Panel C shows a super shift assay with the antiserum against synthetic PREB. Lane 1, probe A1 only; lane 2, synthetic PREB was added; lane 3, synthetic PREB with 5 µl of pre-immune serum was added, whereas 5 µl of antiserum raised against PREB was added. Arrow points to the probe A1-specific complex.

Figure 6. PREB is involved in cAMP suppressed adiponectin expression

A) SiRNA of PREB (si-PREB) or control siRNA (cont) was transfected into 3T3-L1 cells. cAMP treatment was performed the following day. At 72 h after transfection, the expression of adiponectin was measured using Western blot analysis. The ratio of adiponectin to GAPDH is shown as a percentage of control. Each data point shows the mean ± SE (n=3) of separate experiments. The asterisk denotes a significant difference (P<0.05). B) Cells were transfected with the reporter genes containing -984 WT LUC, -984 mA1 LUC, -984 mA2 LUC (data not shown), and -984 mA3 LUC (data not shown); after cAMP treatment, the promoter activity was determined by luciferase assay. All assays were corrected for β-galactosidase activity and total amounts of protein per reaction were identical. The results are expressed as luciferase activities relative to those of control cells arbitrarily set at 100. Each data point shows mean ± SE (n=3) of separate transfections (P<0.05).
Fig. 1A

310bp PREB
281bp

β-actin

Fig. 1A
76x31mm (300 x 300 DPI)
Fig. 1C
69x32mm (300 x 300 DPI)
Fig. 1D

PREB/β-Tubulin (% of control)

undi3T3-L1 di3T3-L1

*
Fig2A
50x39mm (300 x 300 DPI)
Fig 2B
232x101mm (300 x 300 DPI)
C

Fig2C
135x140mm (300 x 300 DPI)
Fig3
149x180mm (300 x 300 DPI)
Fig 4

Mouse adiponectin promoter

-984  +1

A1 A2 A3

A1 (-398 to -395)
A2 (-324 to -321)
A3 (-285 to -273)

Relative promoter activity (% of control)

control  PREB  control  PREB  control  PREB  control  PREB

Wild type  mA1  mA2  mA3

*  N.S.  *  *

149x150mm (300 x 300 DPI)
Fig. 5
148x61mm (300 x 300 DPI)
A

<table>
<thead>
<tr>
<th></th>
<th>si-Cont</th>
<th>si-PREB</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adiponectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 6A
75x100mm (300 x 300 DPI)
**B**

![Bar graph showing relative promoter activity](image)

- cont
- cAMP ApN
- cAMP mApN1

**Fig6B**

219x166mm (300 x 300 DPI)