Overexpression and Ribozyme-mediated Targeting of Transcriptional Coactivators CREB-binding Protein and p300 Revealed Their Indispensable Roles in Adipocyte Differentiation through the Regulation of Peroxisome Proliferator-activated Receptor γ*

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The cAMP-response element-binding protein-binding protein (CBP) and p300 are common coactivators for several transcriptional factors. It has been reported that both CBP and p300 are significant for the activation of peroxisome proliferator-activated receptor γ (PPARγ), which is a crucial nuclear receptor in adipogenesis. However, it remains unclear whether CBP and/or p300 is physiologically essential to the activation of PPARγ in adipocytes and adipocyte differentiation. In this study, we investigated the physiological significance of CBP/ p300 in NIH3T3 cells transiently expressing PPARγ and CBP and in 3T3-L1 preadipocytes stably expressing CBP- or p300-specific ribozymes. In PPARγ-transfected NIH3T3 cells, induction of expression of PPARγ target genes such as adipocyte fatty acid-binding protein (aP2) and lipoprotein lipase (LPL) by adding thiazolidinedione was enhanced, depending on the amount of a CBP expression plasmid transfected. Expression of aP2 and LPL genes, as well as glycerol-3-phosphate dehydrogenase activity and triacylglyceride accumulation after adipogenic induction, was largely suppressed in 3T3-L1 adipocytes expressing either the CBP- or p300-specific active ribozyme, but not in inactive ribozyme-expressing cells. These data suggest that both CBP and p300 are indispensable for the full activation of PPARγ and adipocyte differentiation and that CBP and p300 do not mutually complement in the process.

Adipose tissues are significant in regulation of common diseases such as obesity, type 2 diabetes, coronary artery disease, and hypertension (1). This is because, during their differentiation and maturation, adipocytes release many bioactive molecules (called “adipocytokines”), including adipin, angiotensinogen, leptin, tumor necrosis factor α (TNF-α),1 and adiponectin (2). TNF-α is a negative factor released from mature adipocytes, that is, it suppresses glucose uptake into adipose tissues or skeletal muscles (3). On the other hand, adiponectin is a positive factor released from nonmature adipocytes, that is, it enhances insulin sensitivity (4, 5). Thus, understanding the mechanism underlying adipocyte differentiation is essential to management of common diseases.

Adipocyte differentiation is a complex process regulated by various factors. Upon induction of differentiation, a cascade of gene transcription events occurs, leading to the expression of adipocyte-specific genes (6). One of the essential genes involved in the cascade encodes peroxisome proliferator-activated receptor γ (PPARγ), a member of the ligand-activated nuclear receptor superfamily (7). PPARγ binds to the retinoid X receptor (RXR) (8) and up-regulates the expression of adipocyte-specific genes to promote adipocyte differentiation (9). Exogenous expression of PPARγ transforms NIH3T3 fibroblasts and G5 myoblasts into adipocyte-like cells (10, 11). Moreover, PPARγ is activated by anti-diabetes drugs, such as thiazolidinediones (TZDs) (12). TZDs stimulate differentiation of preadipocytes and up-regulate glucose uptake into the adipose tissue by activating PPARγ. TZDs also suppress the expression of TNF-α and enhance that of adiponectin in differentiated adipocytes (13, 14). Therefore, activation of PPARγ is involved in the regulation of adipocyte differentiation as well as insulin activity in adipose tissues.

It has recently been reported that coactivators are necessary for the activation of nuclear receptors, including PPARγ (15, 16). Coactivators interact with nuclear receptors in a ligand-dependent manner and recruit basal transcriptional factors

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1 The abbreviations used are: TNF-α, tumor necrosis factor α; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; TZD, thiazolidinedione; DM, differentiation medium; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; RXR, retinoid-X receptor; C/EBP, CCAAT/enhancer-binding protein; LPL, lipoprotein lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
such as RNA polymerases proximal to a nuclear receptor complex in a gene promoter region. Among the coactivators, the cAMP-response element-binding protein (CREB)-binding protein (CBP) and its highly related p300 protein have been rather well characterized to date. These coactivators are expressed ubiquitously, and they participate in many basic cellular events (17). PPARγ interacts with CBP and p300 in a ligand-dependent manner, and p300, in turn, enhances the activity of PPARγ (18, 19). However, it has not yet been clarified whether CBP and/or p300 can actually affect the expression of PPARγ target genes in adipocytes or whether expression of endogenous CBP and/or p300 is indispensable for adipocyte differentiation.

The aim of this study was to elucidate the physiological role of CBP and p300 in PPARγ-mediated gene expression in preadipocytes and adipocytes. Detailed analyses revealed that overexpression of CBP or p300 with PPARγ enhanced the expression of PPARγ target genes in NIH3T3 cells. Moreover, either CBP- or p300-specific ribozyme-mediated targeting resulted in suppressed expression of adipogenic markers such as adipocyte fatty acid-binding protein (aP2) and lipoprotein lipase (LPL), as well as reduction in glycerol-3-phosphate dehydrogenase activity and lipid accumulation in 3T3-L1 cells upon induction of adipocyte differentiation. This suggests that both CBP and p300 are indispensable for adipocyte differentiation and that CBP and p300 do not mutually complement in the process. To our knowledge, this is the first report of the physiological relevance of CBP and p300 in adipocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—T174 TZD, a specific ligand for PPARγ (18), was kindly provided by Tanabe Seiyaku Co., Ltd. (Osaka, Japan). All other chemicals were from Sigma or Nacalai Tesque (Kyoto, Japan) and were of guaranteed grade or tissue culture grade.

**Plasmid Construction and Preparation of Recombinant Retrovirus**—Expression plasmids for coactivators, pCMX-CBP and pCMX-p300, were kindly gifts from Dr. R. H. Goodman (Oregon Health Sciences University) and Dr. D. M. Livingston (Harvard Medical School), respectively. pSG5-CBP, which included CBP cDNA in pBlueScript-SK(+) (Stratagene), was used as a control plasmid to make the amounts of CBP and p300-specific ribozymes—RzCBP-mut and Rzp300-mut, respectively. A luciferase reporter plasmid containing four tandem repeats of the PPAR response element (PPRE) followed by a thymidine kinase (TK) promoter, p4xPPRE-tk-luc, was from Dr. K. Umesono (Kyoto University) and Dr. D. M. Livingston (Harvard Medical School), respectively. pRL-CMV (Promega) was used as an internal control to normalize transfection efficiencies in luciferase assays. CBP- and p300-specific active ribozymes (RxCBPEP4wt and Rzp300wt, respectively) have target sequences against human nucleic acid sequences 484–502 in pCB cDNA and 364–382 in p300 cDNA, respectively (20). Inactive mutants of the CBP- and p300-specific ribozymes (RxCBPEP4mut and Rzp300mut, respectively) have point mutations on each ribozyme active site, which cannot cleave target mRNAs (20).

Fragments of RxCBPEP4 and Rzp300 with EcoRI and Sall ends were inserted into a retrovirus expression vector, pMX-puro (a gift from Dr. T. Kitamura, University of Tokyo) (21) via the same sites, generating pMX-RxCBPEP4 and pMX-Rzp300, respectively. For the preparation of recombinant retroviruses, expression constructs were transiently transfected into Phoenix ecotropic packaging cells (a kind gift from Dr. G. Nolan, Stanford University) using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol, and then the conditioned medium was recovered for subsequent infection.

**Cell Culture**—Murine NIH3T3 fibroblasts and murine 3T3-L1 preadipocytes were purchased from American Type Culture Collection. All cell lines were maintained in a maintenance medium (10% fetal bovine serum, 10% serum-free medium, 10% 15 mM NaCl, 10 mM KCl, 10 mM KH₂PO₄, 10 mM Na₂HPO₄, 10 mM NaHCO₃, 10 mM HEPES, and 1% Penicillin/Streptomycin in Dulbecco’s modified Eagle’s medium) at 37 °C in 5% CO₂/95% air under a humidified condition. For luciferase assays using NIH3T3 cells cultured on 24-well tissue culture plates, pSG5-mPPARγ (0.4 μg/well), pCMX-CBP and/or pSK-CBP (0.4 μg/well), p4xPPRE-tk-luc (0.4 μg/well), and pRL-CMV (0.4 μg/well) were transfected into NIH3T3 cells. For quantification of PPARγ target transcripts, pSG5-mPPARγ (0.5 μg/well) and pCMX-CBP and/or pSK-CBP (0.5 μg/well) were transfected into NIH3T3 cells cultured on 6-well tissue culture plates. An expression vector for E1A, pE1A (0.5 μg/well), was included as indicated in Fig. 1B to inhibit CBP/p300 activity. The transfections were performed using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. Twenty-four hours after transfection, cells were supplemented with 10 μM TZD, cultured for another 24 h, and then lysed in the recommended lysis buffer for estimation of luciferase activity or harvested for mRNA preparation. Luciferase assays were performed using the dual luciferase assay system (Promega).

3T3-L1 cells expressing RzCBP-wt-mut or Rzp300-wt-mut were seeded into 12-well tissue culture plates containing 2.0 ml of adipocyte medium. After 48 h, the cell culture medium was changed to post-DM, which was DM supplemented with 5 μg/ml insulin, and then the medium was replaced with fresh medium every 2 days. Eight days after differentiation induction, the cells were washed with phosphate-buffered saline, and total RNA was prepared using an RNeasy minikit (Qagen, Hilden, Germany) according to the manufacturer’s protocol. To exclude the clonal variation in adipocyte differentiation, polyclonal cells were used directly for subsequent assays. The cells expressing ribozymes were cultured on 6-well tissue culture plates for immunoblotting and differentiation assays as described previously (22). Briefly, after 4 days, when confluence was reached, cells were incubated in a differentiation medium (DM), which is the maintenance medium supplemented with 0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methylxanthine. After 40 h, the cell culture medium was changed to post-DM, which is DM supplemented with 5 μg/ml insulin and then the medium was replaced with fresh medium every 2 days. Eight days after differentiation induction, the cells were washed with phosphate-buffered saline, and total RNA was prepared using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. To exclude the clonal variation in adipocyte differentiation, polyclonal cells were used directly for subsequent assays.

**Biochemical Assays, Immunoblotting, and Oil-Red O Staining**—Samples for biochemical assays were prepared using cells cultured on 6-well tissue culture plates. The measurement of glycerol-3-phosphate dehydrogenase activity was performed as described previously (22). The content of cellular triglyceride was measured using a TG Test Wako kit (Wako Pure Chemical Industry Ltd., Osaka, Japan). Protein concentrations of samples for immunoblotting were determined using a protein assay kit (Bio-Rad). Immunoblotting was carried out using an enhanced chemiluminescence system (PerkinElmer Life Sciences) as described previously (22). The anti-mouse PPARγ antibody was obtained from Affinity Bioreagents, Inc., and antibodies against RXXrs, C/EBPs, C/EBPβ, C/EBPδ, and p300 were from Santa Cruz Biotechnology Inc. Anti-β-actin and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs were purchased from Chemicon International Inc. and DAKO A/S (Copenhagen, Denmark), respectively. Oil-Red O staining was performed as follows: cells were washed with phosphate-buffered saline and then stained with 60% filtered Oil-Red O stock solution (0.15 g of Oil-Red O in 50 ml of isopropanol) for 30 min at 37 °C. The nonwashed samples were washed with phosphate-buffered saline and then washed briefly with water and examined under a microscope.

**mRNA Preparation and Quantification**—Aliquots of total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and a thermal cycler (Takara PCR Thermal Cycler SP; Takara Shuzo Co., Kyoto, Japan) according to the manufacturer’s instructions. To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (LightCycler System; Roche Diagnostics). The oligonucleotide primer sets of mouse PPAR target genes were designed using a PCR primer selection program at the web site of the Virtual Genomic Center from the GenBank™ database as follows: (a) mouse LPL (GenBank™ accession number J03302), forward primer 5’-ATCCATGGTGACGGTGTTAAGC-3’ and reverse primer 5’-CTGGATCCGACCATACTGACCA3’; (b) p2 (GenBank™ accession number K02109), forward primer 5’-AAGACAGCTTCTTGAGAAGTT-3’ and reverse primer 5’-TGACAAATCCTCCATTGAGC3’; and (c) glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank™ accession number M32599), forward primer 5’-GAAGGTCGGTGACCAAGGTG-3’ and reverse primer 5’-GAGGACGAGCTTCTTGAGAAGTT-3’. Amplification was performed according to a published protocol (23). Briefly, the reaction solution (10 μl, final volume) contained 3 μM MgCl₂, 2.0 μl of LightCycler DNA Master SYBR Green I dye, and 5 μl of each primer. The standard amplification program included 30 cycles of three steps each, which involved heating the product to 95 °C at 20 °C/s with a 30-s hold, annealing to 55 °C at 20 °C/s with a 10-s hold, and extension to 72 °C at 20 °C/s with a 10-s hold. The fluorescence at 530 nm was recorded on-line at the end of the extension step. The amount of standard plasmid was calculated from the absorbance at 260 nm and the molecular mass of each plasmid. The copy numbers of standards and samples were amplified simultaneously in the LightCycler. The first cycle number indicated specific fluorescence against noise, and the logarithm of the concentration of the PCR product standard.
ard, the external standard curve, was calculated using LightCycler software. To confirm the amplification of specific transcripts, melting curve profiles were generated at the end of each run. To compare the mRNA expression level among samples, the copy number of each transcript was divided by that of GAPDH, which showed a constant mRNA expression level. All data indicating the mRNA expression level were presented as a ratio with respect to that of the control in each experiment.

Statistical Analysis—The data are presented as means ± S.E. and were analyzed statistically using the unpaired t test or the Welch t test when variances were heterogeneous. Differences were considered significant at *p < 0.05.

RESULTS

Increase in Expression Level of CBP/p300 Proteins Enhances the Expression of PPARγ Target Genes in Intact Cells—It has been reported in detail that CBP and p300 interact with PPARγ in a ligand-dependent manner, and increasing the amount of p300 enhanced PPARγ activity (18, 19). However, it remained unknown whether an increase in the CBP protein expression level could promote PPARγ transactivation in intact cells. To elucidate this, we performed luciferase assays by transfecting a reporter plasmid with the PPRE into PPARγ-transfected NIH3T3 fibroblasts, which differentiate into adipocyte-like cells in a PPARγ ligand-dependent manner (10).

Increasing the amount of an expression plasmid for enhanced luciferase activity in the presence of 10 μM TZD (Fig. 1A). Cotransfection of 0.2, 0.4, and 0.8 μg/well pCMX-CBP (a CBP expression vector) induced luciferase activities that were 1.2-, 1.9-, and 2.8-fold higher, respectively, than those of mock transfectants in the presence of TZD. These results suggest that the expression of CBP could enhance PPARγ activity against ligand as much as expression of p300.

It was also investigated whether coexpression of CBP/p300 could regulate the gene promoter activity of endogenous promoters of PPARγ target genes in cells. We used the PPARγ-transfected NIH3T3 cells for luciferase assays. As shown in Fig. 1B, cotransfection of PPARγ and CBP expression plasmids up-regulated the expression of endogenous aP2 (left panel) and LPL (right panel) by the addition of 10 μM TZD. PPARγ regulates the expression of aP2 and LPL, which is parallel to adipocyte differentiation (24, 25). Therefore, aP2 and LPL have been used as well-characterized PPARγ target genes and typical adipocyte differentiation markers. The expression of these endogenous genes depended on the amount of CBP plasmids transfected; cotransfection of 0.5 and 1.0 μg/well pCMX-CBP resulted in 2.3- and 4.1-fold increases in the up-regulation of aP2, respectively, compared with mock transfectants in the presence of 10 μM TZD (Fig. 1B, left panel). In a similar manner, cotransfection of 0.5 and 1.0 μg/well pCMX-CBP increased the expression level of LPL by 1.5- and 2.0-fold, respectively, compared with mock transfectants (Fig. 1B, right panel).

Nearly the same results were obtained for p300 coexpression (data not shown). Moreover, the enhancement by CBP coexpression was significantly suppressed by coexpression of adenoviral oncprotein E1A, which is known as a viral regulatory protein that specifically suppresses CBP/p300 activity in virus-infected cells (26) (Fig. 1B). Therefore, this result indicates that the increase in the CBP expression level was involved in the up-regulation of PPARγ target genes in cells.

CBP or p300 Targeting Specific Ribozymes Inhibits the Expression of PPARγ Target Genes in 3T3-L1 Precadipoocytes—Next, to further deepen the understanding of the physiological relevance of CBP and p300, we established 3T3-L1 preadipocytes expressing a CBP- or p300-specific active ribozyme (RzCBP-wt or Rzp300-wt, respectively). RzCBP-wt and Rzp300-wt, which specifically cleave target mRNAs, can down-regulate the expression of CBP and p300 protein, respectively, in cells expressing the ribozyme (20).

FIG. 1. CBP enhances PPARγ activation in NIH3T3 cells. A, NIH3T3 cells were transfected with an expression plasmid for mouse PPARγ (0.4 μg/well), a reporter plasmid with PPRE (0.4 μg/well), and increasing amounts of an expression plasmid for CBP. pRL-CMV (0.8 ng/well) was also included as an internal control to normalize transfection efficiency. Twenty-four h after transfection, cells were incubated in the presence or absence of 10 μM TZD (T174) for 24 h. Cells were lysed, and luciferase activity was assayed as described under “Experimental Procedures.” Relative luciferase activity was presented as fold induction with respect to that of mock transfectants (without CBP) in the absence of TZD. The values are the means ± S.E. of four tests. *, *p < 0.05 compared with mock transfectants. B, NIH3T3 cells were transfected with an expression vector for mouse PPARγ (0.5 μg/well) and increasing amounts of an expression vector for CBP. An expression vector for E1A (0.5 μg/well) was included as indicated. Cells were cultured in the presence or absence of 10 μM TZD (T174) for 24 h after transfection, and then total RNA samples were prepared. The expression levels of aP2 (left panel) and LPL (right panel) were estimated using LightCycler and normalized with respect to the GAPDH expression level (each copy number of aP2 and LPL was divided by that of GAPDH). The relative gene expression is presented as the ratio of the expression level of a gene to that of the vehicle control without transfection of the CBP expression vector. The values are the means ± S.E. of six tests. **, *p < 0.05 compared with a sample cotransfected without a CBP expression vector. ***, *p < 0.05 compared with a sample cotransfected without an E1A expression vector.
ever, there was no difference in the expression levels of p300 between 3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut (Fig. 2). Conversely, the p300 expression level in 3T3-L1-Rzp300-wt decreased ~30% as compared with that in 3T3-L1-Rzp300-mut (Fig. 2, right panels). Steroid receptor coactivator 1 (SRC-1), another coactivator for PPARγ(27), and β-actin were expressed at nearly the same levels in all cells used. These results indicate that ribozymes specifically decreased the CBP or p300 mRNA expression level, resulting in a decreased expression level of each protein in 3T3-L1 preadipocytes.

We next examined the expression levels of transcription factors involved in PPARγ activation in 3T3-L1-RzCBP-wt-mut. Confluent cells were lysed and separated by SDS-PAGE followed by immunoblotting. As shown in Fig. 3A, the expression level of PPARγ in 3T3-L1-RzCBP-wt was comparable to that in 3T3-L1-RzCBP-mut (Fig. 3A). Although PPARγ, another isoform of PPARγ in preadipocytes, was not detected, the total expression levels of PPARγ and PPARγ were shown to be comparable in 3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut by quantitative reverse transcription-PCR analyses (data not shown). RXRs are heterodimer partners of PPARγ and are essential to various functions of PPARγ (28). In 3T3-L1 cells, RXRα is a functional subtype, and RXRβ is expressed as well (8, 30). The expression levels of RXRα also did not differ in 3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut (Fig. 3A). The absence of difference in the PPARγ and RXRα expression levels in undifferentiated cells suggests that a decreased expression level of CBP does not affect the basal expression levels of PPARγ and RXRα in 3T3-L1 preadipocytes.

3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut were then stimulated with TZD, and the expression of PPARγ target genes aP2 and LPL was investigated. The addition of 10 μM TZD resulted in a 5.3- and 5.4-fold increase in the expression level of aP2 and LPL mRNA, respectively, in 3T3-L1-RzCBP-wt, whereas only a 2- and 1.4-fold increase in aP2 and LPL mRNA, respectively, was observed in 3T3-L1-RzCBP-mut (Fig. 3B). Experiments using 3T3-L1-Rzp300-wt-mut showed results similar to those using 3T3-L1-RzCBP-wt-mut (data not shown). These data suggest that the decrease in expression levels of CBP/p300 resulted in the suppression of PPARγ target gene expression in 3T3-L1 preadipocytes and that endogenous expression of CBP/p300 was essential to the induction of PPARγ target genes in 3T3-L1 preadipocytes.

**Targeting of CBP or p300 by Specific Ribozymes Inhibits Adipocyte Differentiation in 3T3-L1 Preadipocytes**—Finally, we investigated whether the decrease in the expression level of endogenous CBP or p300 in 3T3-L1 preadipocytes could affect their differentiation into adipocytes. 3T3-L1-RzCBP-wt-mut and 3T3-L1-Rzp300-wt-mut were cultured in DM for 40 h and then cultured in post-DM. Eight days after differentiation induction, 3T3-L1-RzCBP-mut accumulated fat droplets in cells (Fig. 4A, a). On the other hand, 3T3-L1-RzCBP-wt showed a low level of accumulation of fat droplets (Fig. 4A, b). Essentially the same results were obtained in Oil-Red O staining, by which triacylglycerides were stained red (Fig. 4A, a, c, and d). To confirm the low level of accumulation of lipid droplets, we determined the triacylglyceride level in the cells. As shown in Fig. 4A, e, 8 days after differentiation induction, the triacylglyceride content in 3T3-L1-RzCBP-wt cells was significantly lower than that in 3T3-L1-RzCBP-mut cells. As observed in 3T3-L1-RzCBP-wt, the lipid levels in 3T3-L1-Rzp300-wt were also significantly lower than that in 3T3-L1-Rzp300-mut (Fig. 4B). Moreover, glycerol-3-phosphate dehydrogenase activity, which is one of the biochemical markers of adipocyte differentiation, was also significantly suppressed in 3T3-L1-RzCBP-wt and in
The values are the means ± S.E. of six independent tests. *p < 0.05 compared with the inactive ribozyme controls.

3T3-L1-Rzp300-wt throughout the course of adipocyte differentiation (Fig. 5). Therefore, it was shown that the decrease in the expression level of endogenous CBP or p300 in the presence of the active ribozymes resulted in the suppression of 3T3-L1 preadipocyte differentiation.

Eight days after differentiation induction, the expression levels of aP2 and LPL genes, which are PPARγ target genes and adipocyte differentiation markers, were estimated by quantitative real-time reverse transcription-PCR method. As shown in Fig. 6A, the expression level of aP2 in 3T3-L1-RzCBP-wt was lower than that in 3T3-L1-RzCBP-mut (Fig. 6A, left panel). The expression of LPL was also significantly suppressed, but the extent of suppression was smaller (Fig. 6A, right panel). The suppressed expression of aP2 and LPL genes in 3T3-L1-Rzp300-wt was the same as that in 3T3-L1-RzpCB-wt (data not shown).

Although the expression of aP2 and LPL is regulated mainly by PPARγ with respect to the differentiation of 3T3-L1 cells, the C/EBP family, as well as PPARγ, is also known to be involved in the regulation of adipocyte differentiation (31). Therefore, the expression of the C/EBP family, as well as PPARγ, in the early and late phases of adipocyte differentiation was examined by immunoblotting. Eight days after the differentiation induction in 3T3-L1-RzCBP-mut, the PPARγ expression level was about 3.5-fold higher than that on day 0 (the start of differentiation induction) (Fig. 6B, top panels). However, the expression level of PPARγ in 3T3-L1-RzCBP-wt was lower than that in 3T3-L1-RzCBP-mut (−65%). PPARγ was not detected by the antibody used, but the expression of PPARγ mRNA was also suppressed in differentiated 3T3-L1-RzCBP-wt (data not shown). The expression levels of the full-length 42-kDa C/EBPα in 3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut were nearly comparable 2 days after
diffusion induction. Eight days after differentiation induction, an ~3-fold increase in the expression level of C/EBPα was induced in 3T3-L1-RzCBP-mut, whereas only about a 1.5-fold increase in the C/EBPα expression level was induced in 3T3-L1RzCBP-wt (Fig. 6, second panel from the top). On the other hand, there was no obvious difference in the expression level of C/EBPδ and C/EBPβ/liver activator protein (LAP) (32-kDa form), which were thought to be regulators of PPARγ and C/EBPα induction (31) (Fig. 6B, third and fourth panels from the top). Other isoforms of C/EBPs, such as 30-kDa C/EBPα and 18-kDa C/EBPβ/liver inhibitory protein (LIP), exhibited nearly the same expression profiles as 42-kDa C/EBPα and 18-kDa C/EBPβ/LAP, respectively (data not shown). These results suggest that the inhibition of adipocyte differentiation by the decrease in C/EBP expression is due primarily to the suppression of PPARγ expression and activity, but other transcriptional factors such as C/EBPα could be involved in the differentiation process.

DISCUSSION

In this study, we showed that the increase in the expression level of CBP, a coactivator for PPARγ, resulted in the activation of PPARγ in NIH3T3 cells by targeting PPRE in luciferase reporter plasmid (Fig. 1A) and endogenous promoters of aP2 and LPL genes (Fig. 1B). NIH3T3 fibroblasts are not preadipocytes, but exogenous expression of PPARγ transforms the transfected NIH3T3 cells into preadipocytes, which can differentiate into adipocytes by treatment with a combination of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin or TZD (10). To our knowledge, our results first showed that PPARγ ligand-dependent expression of PPARγ target genes in intact cells, as in NIH3T3 fibroblasts, was induced by the ectopic expression of CBP or p300. This suggests that the expression of endogenous CBP/p300 could be a rate-limiting factor in PPARγ activation in NIH3T3 cells.

The physiological significance of CBP/p300 in complete activation of PPARγ was further examined in ribozyme-mediated targeting experiments. Decreasing the CBP or p300 expression level in 3T3-L1 preadipocytes using specific ribozymes suppressed PPARγ ligand-dependent induction of aP2 and LPL (Fig. 3). This suggests that the expression levels of both CBP and p300 are indispensable for induction of PPARγ target genes in 3T3-L1 preadipocytes. Moreover, the expression level of endogenous CBP or p300 was essential for differentiation of 3T3-L1 preadipocytes (Figs. 4 and 5). Although CBP and p300 share high sequence similarity throughout their entire structure (32), several differences in their functions have been reported (20, 33), suggesting that CBP and p300 might function at different points in the course of adipocyte differentiation. It is known that many nuclear transcriptional factors such as PPARs and C/EBPs are involved in adipocyte differentiation and that the activation of those transcriptional factors requires several coactivators (or a coactivator complex), including CBP/p300 (18, 19), steroid receptor coactivator 1 (27), and PPARγ coactivators (34, 35). Inhibition of one or more steps of transcriptional regulation in adipocyte differentiation by a decrease in either CBP or p300 expression could totally suppress the transcriptional cascade, leading to the inhibition of adipocyte differentiation. Another possibility is that CBP and p300 might function equally, and the total expression level of CBP and p300 is essential to the complete activation of PPARγ and adipocyte differentiation. In this sense, it might be interesting to examine whether ectopic expression of CBP or p300 in 3T3-L1-RzCBP-wt or 3T3-L1-RzCBP-wt could complement the suppression of PPARγ activity and adipocyte differentiation.

Although our experiments focused mainly on PPARγ in preadipocytes and adipocytes, the C/EBP family is also important in adipocyte differentiation (36). The following model is widely accepted. C/EBPβ and C/EBPδ are induced early and temporally in adipocyte differentiation, and then they stimulate PPARγ and C/EBPα expression. Finally, PPARγ and C/EBPα induce their mutual expressions under the control of CBP and p300 coactivators (Refs. 37 and 38; namely, there is a positive feedback loop between PPARγ and C/EBPα (39) and synergistically promote adipocyte differentiation. Targeting of CBP by RzCBP-wt resulted in suppression of PPARγ and C/EBPα expression, whereas C/EBPβ and C/EBPδ were expressed at comparable levels (Fig. 6). These data suggest that CBP/p300 are necessary for induction of PPARγ and C/EBPα expression, but not for that of C/EBPβ or C/EBPδ. The decrease in the PPARγ and C/EBPα expression might be due to decreased activities of C/EBPβ and C/EBPδ because CBP can act as a coactivator for the transcriptional factors (40, 41). Alternatively, there might be a mechanism independent of C/EBPβ and C/EBPδ activation that regulates PPARγ and C/EBPα expression, as Akira and co-workers reported previously (42). They proposed an alternative mechanism that regulates PPARγ and C/EBPα expression because PPARγ and C/EBPα expression was normal in mice lacking C/EBPβ and C/EBPδ. Thus, it is suggested that CBP and p300 function sequentially in both activation and expression of transcriptional factors involved in the adipocyte differentiation process.

The expression levels of PPARγ in undifferentiated 3T3-L1-RzCBP-wt and 3T3-L1-RzCBP-mut cells were apparently the same (Fig. 3), suggesting that CBP and/or p300 is not necessary in the basal expression of PPARγ, although they were essential in the differentiation-dependent induction of PPARγ. This might be because other coactivators compensated for the function of CBP/p300 in basal expression of PPARγ or because the basal expression was regulated by a possible mechanism that is independent of CBP/p300.

Our data showing that the expression of CBP and p300 was indispensable for complete activation of PPARγ and adipocyte differentiation suggest that dysfunction of CBP and/or p300 might be associated with common diseases such as obesity and diabetes. A recent study showed that the interaction of PPARγ with distinct coactivators was ligand type-specific (43), suggesting that PPARγ target genes could be regulated by various combinations of coactivators and PPARγ ligands. In the present study, a decrease in the CBP expression level suppressed gene expression of aP2 more strongly than that of LPL in differentiated 3T3-L1 cells (Fig. 6), and this was somehow consistent with the observation that the overexpression of CBP in NIH3T3 cells up-regulated aP2 expression more strongly than LPL expression (Fig. 2). Thus, CBP/p300 might be more significant in aP2 gene expression in PPARγ-transfected NIH3T3 cells and in 3T3-L1 adipocytes. The expression of aP2 in adipose tissues links obesity to insulin resistance. Obese aP2 knockout mice did not develop insulin resistance and diabetes due to failure in TNF-α expression in adipose tissues (44). Expression of aP2 is central to the pathway that links obesity to insulin resistance by linking fatty acid metabolism to TNF-α expression. With respect to the significance of CBP in aP2 expression, we propose that CBP might be a good candidate for treatment of obesity and diabetes. This is supported by our preliminary data showing that the CBP expression level in adipose tissues of KK-Ay strain mice (obesity and diabetes model mice) was higher than that of A/J strain mice (obesity resistance model mice), although the expression levels of other coactivators such as SRC-1 were almost the same.2 In this sense, it is interesting to elucidate how CBP expression is

2 T. Kawada and N. Takahashi, manuscript in preparation.
regulated in adipocytes. Abnormality in CBP expression will result in critical damage to many tissues as well as adipose tissues because CBP is ubiquitously expressed and is essential to basic cellular functions (17), and it has been shown that disruption of the mouse CBP gene was lethal to the embryo (45). Furthermore, CBP dysfunction caused Rubinstein-Taybi syndrome (46). Recently, such diseases have been called “coactivator diseases” (29), which are characterized by severe generalized dysfunctions. Thus, we again emphasize the physiological relevance of CBP/p300 in adipocyte differentiation and lipid metabolism.

PPARγ plays a central role in adipocyte differentiation and lipid metabolism by adipocytes. Understanding the mechanisms by which PPARγ is activated leads to effective management of common diseases including obesity, diabetes, and atherosclerosis. PPARγ activation is regulated mainly in a ligand-dependent manner. However, because the interaction of PPARγ with coactivators is also important in adipocyte differentiation and is regulated in a ligand-type-specific manner as discussed above, the regulation of PPARγ activation and its own expression by coactivators may be the primary system. In this regard, our study is very important not only in investigating adipocyte differentiation but also in clarifying the relationship between coactivators and common diseases such as obesity and diabetes.

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