Adipocyte Determination- and Differentiation-dependent Factor 1/Sterol Regulatory Element-binding Protein 1c Regulates Mouse Adiponectin Expression

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Jong Bae Seo‡‡, Hyang Mi Moon‡, Mun Ju Noh‡, Yun Sok Lee‡, Hyun Woo Jeong‡‡, Eung Jae Yoo‡, Woo Sik Kim‡‡, Jiyoung Park‡‡, Byung-S. Youn‡, Jae Woo Kim**

From the ‡School of Biological Sciences, Seoul National University, Seoul, Korea 151-742, *KOMED Institute for Life Science, Korea University, Seoul, Korea 136-701, **Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Yonsei University College of Medicine, Seoul 120-752, Korea, and ‡‡International Vaccine Institute, Seoul, Korea 151-818

Adiponectin is exclusively expressed in differentiated adipocytes and plays an important role in regulating energy homeostasis, including the glucose and lipid metabolism associated with increased insulin sensitivity. However, the control of adiponectin gene expression in adipocytes is poorly understood. We show here that levels of adiponectin mRNA and protein are reduced in the white adipose tissue of ob/ob and db/db mice and that there is a concomitant reduction of the adipocyte determination- and differentiation-dependent factor 1 (ADD1/SREBP1c) transcription factor. To determine whether ADD1/SREBP1c regulates adiponectin gene expression, we isolated and characterized the mouse adiponectin promoter. Analysis of the adiponectin promoter revealed putative binding sites for the adipogenic transcription factors ADD1/SREBP1c, peroxisomal proliferator-activated receptor γ and CCAAT enhancer-binding proteins. DNase I footprinting and chromatin immunoprecipitation analyses revealed that ADD1/SREBP1c binds in vitro and in vivo to the proximal promoter containing sterol regulatory element (SRE) motifs. A luciferase reporter containing the promoter was transactivated by ADD1/SREBP1c, and introduction of SRE mutations into the construct abolished transactivation. Adenoviral overexpression of ADD1/SREBP1c also elevated adiponectin mRNA and protein levels in 3T3-L1 adipocytes. Furthermore, insulin stimulated adiponectin mRNA expression in adipocytes and augmented transactivation of the adiponectin promoter by ADD1/SREBP1c. Taken together, these data indicate that ADD1/SREBP1c controls adiponectin gene expression in differentiated adipocytes.

Adiponectin is a protein that is exclusively expressed in differentiated adipocytes and plays an important role in regulating energy homeostasis, including the glucose and lipid metabolism associated with increased insulin sensitivity. However, the control of adiponectin gene expression in adipocytes is poorly understood. We show here that levels of adiponectin mRNA and protein are reduced in the white adipose tissue of ob/ob and db/db mice and that there is a concomitant reduction of the adipocyte determination- and differentiation-dependent factor 1 (ADD1/SREBP1c) transcription factor. To determine whether ADD1/SREBP1c regulates adiponectin gene expression, we isolated and characterized the mouse adiponectin promoter. Analysis of the adiponectin promoter revealed putative binding sites for the adipogenic transcription factors ADD1/SREBP1c, peroxisomal proliferator-activated receptor γ and CCAAT enhancer-binding proteins. DNase I footprinting and chromatin immunoprecipitation analyses revealed that ADD1/SREBP1c binds in vitro and in vivo to the proximal promoter containing sterol regulatory element (SRE) motifs. A luciferase reporter containing the promoter was transactivated by ADD1/SREBP1c, and introduction of SRE mutations into the construct abolished transactivation. Adenoviral overexpression of ADD1/SREBP1c also elevated adiponectin mRNA and protein levels in 3T3-L1 adipocytes. Furthermore, insulin stimulated adiponectin mRNA expression in adipocytes and augmented transactivation of the adiponectin promoter by ADD1/SREBP1c. Taken together, these data indicate that ADD1/SREBP1c controls adiponectin gene expression in differentiated adipocytes.

Adipose tissue is vital for maintaining whole body energy homeostasis. It has traditionally been considered a mere energy depot, synthesizing and storing triglycerides during periods of caloric excess and releasing free fatty acids and glycerol during periods of nutritional deprivation. However, it is now recognized to play a more active role in regulating glucose and lipid metabolism as an endocrine organ secreting biologically active molecules, so called adipocytokines, that act on the central nervous system and peripheral tissues (1).

Adiponectin (also known as Acrp30, AdipoQ, apM1, and GBP28) is a member of the adipokine family that is exclusively expressed in adipocytes (2–6). The mouse adiponectin gene encodes 247 amino acids with two structurally distinct domains: an N-terminal collagen-like fibrous domain and a complement C1q-like globular domain at the C terminus (3). Adiponectin forms oligomers and seems to circulate in the plasma as a homotrimer or as larger complexes of 12 to 15 subunits. One of its most interesting features, observed in rodents, monkeys, and humans, is that its expression in adipose tissue and plasma is reduced in obese and diabetic subjects (3, 7–11). Adiponectin increases the insulin sensitivity associated with activation of insulin signaling and glucose uptake (12, 13). Furthermore, administration of full-length adiponectin lowers plasma glucose levels by suppressing hepatic glucose production in obese and diabetic mice (14). In addition, the globular domain of adiponectin reduces elevated fatty acid levels in skeletal muscle by stimulating fatty acid oxidation (13, 15). These insulin-sensitizing effects seem to be mediated by AMP kinase (13, 15). Although adiponectin-deficient mice are neither obese nor insulin-resistant under basal conditions, they exhibit elevated levels of insulin, free fatty acids, and glucose, together with insulin resistance, when placed on a high fat diet (16, 17). Adiponectin is also implicated in protection from atherosclerosis because its expression is reduced in patients with coronary artery disease (18–21). Adiponectin decreases the attachment of monocytes to human aortic endothelial cells, which represents an early stage of atherosclerotic vascular damage, by lowering expression of several adhesion molecules (18, 22). Furthermore, it reduces levels of macrophage scavenger receptors so that they accumulate fewer lipid droplets (19, 23). Yamauchi et al. (24) have cloned two mouse adiponectin receptors, adiponectin receptors 1 and 2, both of which have seven transmembrane domains. They are abundantly expressed in skeletal muscle and liver, respectively (24). It is likely that they serve as receptors for globular and full-length adiponectin, and mediate the increased

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§§ To whom correspondence should be addressed: Sun 56-1, Sillim-Dong, Kwanak-Gu, Seoul, Korea, 151-742, Tel.: 82-2-880-5852; Fax: 82-2-878-5852; E-mail: jaebkim@snu.ac.kr

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AMP kinase and ligand-dependent PPARs activities in response to adiponectin (24).

A number of factors have been reported to be positive or negative effectors of adiponectin expression. For instance, thiazolidinediones, synthetic ligands of PPARγ, increase both adiponectin gene expression in adipocytes and circulating adiponectin levels (25). Insulin and insulin-like growth factor 1 also increase expression of adiponectin, whereas tumor necrosis factor α (TNFα), another adipocytokine, decreases adiponectin gene expression, suggesting a relationship to TNFα-induced insulin resistance (2, 26–28). Adiponectin mRNA expression is also suppressed via protein kinase A by β-adrenergic stimulation (28, 29). However, there is little information about the transcription factors involved in adiponectin gene expression in adipocytes apart from PPARγ and liver receptor homolog-1 (30).

We report here that the expression of both adiponectin and ADD1/SREBP1c was markedly decreased in the white adipose tissue (WAT) of ob/ob or db/db mouse. To investigate whether ADD1/SREBP1c directly regulates adiponectin expression, we isolated and characterized the mouse adiponectin promoter. Sequence analysis revealed putative binding sites for ADD1/SREBP1c, PPARγ, and C/EBPs, and DNase I footprinting and ChIP assays demonstrated that ADD1/SREBP1c binds to the promoter. Moreover, adenoviral overexpression of ADD1/SREBP1c increased adiponectin expression and secretion in differentiated adipocytes, and this effect was augmented by insulin. These observations indicate that ADD1/SREBP1c is involved in insulin-stimulated adiponectin expression in adipocytes.

EXPERIMENTAL PROCEDURES

Animal Treatment—Male C57BL/6 (10–12 weeks, 18–22 g), ob/ob, and db/db mice were housed five mice/cage, and water was given ad libitum, with a 12-h light-dark cycle beginning at 7:00 a.m. During experiments, food was withdrawn in daylight hours (12 h). There were four C57BL/6 mice in each feeding and fasting group. In the refeeding experiment, food was reintroduced after 12 h of fasting. Animals were sacrificed to isolate epididymal fat.

Northern Blot Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. 20 μg of RNA was denatured in formamide and formaldehyde and separated by electrophoresis on formaldehyde-containing agarose gels. After the RNA was transferred to Nytran membranes, the membranes were cross-linked, hybridized, and washed according to the manufacturer’s recommendations (Schleicher and Schuell). Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (Promega) and [α-32P]dCTP (Amersham Biosciences). CDNA used as probes were as follows: ADD1/SREBP1c, adiponectin, PPARγ, C/EBPα, fatty acid synthase (FAS) and adipocyte fatty acid-binding protein (A-FABP). Western Blot Analysis—Fat tissue was lysed in TQ buffer with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonil fluoride, 500 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, and 10 μg/ml leupeptin. Total cell lysates were centrifuged at 12,000 rpm for 10 min to remove fat debris. Supernatant protein concentrations were determined by the Bio-Rad protein dye reagent. Total extracts (50–80 μg) were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Western blot analysis was performed as recommended by the manufacturer. Mouse adiponectin antibodies were kindly provided by KOMED Inc. (Seoul, Korea) and Dr. T. L. Huh. Bound antibodies were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence and exposure to x-ray film.

DNase I Footprinting—The adiponectin promoter fragments (containing bp –445 to –116) were isolated by double digestion with ApeI and EcoRI to obtain 5′- and 3′-overhanging ends. They were labeled with Klenow fragment and [α-32P]dATP, then purified by PAGE. Binding experiments were performed with 50,000 cpm (1 ng) of probe reannealed in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 7% (ν/ν) glycerol, 1 mM EDTA, 1 mM diethiothreitol, 2 μg of poly (dl-dC) • poly(dl-dC), and the indicated amounts of recombinant ADD1/SREBP1c protein overexpressed in E. coli as described previously (31). After 30 min of incubation on ice, 5 μl of DNase I, freshly diluted in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 25 mM MgCl2, 5 mM trypsin inhibitor, 7% (ν/ν) glycerol was added to the reaction mix, and it was kept at room temperature for 2 min. The dilutions of DNase I ranged from 1:200 to 1:2000 of stock (10 units/μl), depending on the amount of protein in the reaction. Digestion was stopped by the addition of 80 μl of a stop solution containing 20 mM Tris/ HCl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of yeast tRNA, and 10 μg of proteinase K. The samples were incubated for 30 min at 45 °C, extracted with phenol/ chloroform, precipitated with ethanol and resuspended in formamide dye. They were resolved on 6% (ν/ν) polyacrylamide/urea sequencing gels, and the protected regions mapped with reference to the migration of Maxam-Gilbert A+G sequencing products.

Cell Culture—3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (HyClone) and 5% CO2 at 37 °C. Differentiation of preadipocytes to adipocytes was achieved by allowing the cells to reach confluence and adding DMEM supplemented with 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/ml insulin at 5% CO2 and 37 °C. Every 2 days thereafter, fresh medium (DMEM plus 10% fetal bovine serum and 5 μg/ml insulin) was added to the cells. Rat1-IR and human embryonic kidney 293 cells were maintained in DMEM supplemented with 10% (ν/ν) bovine calf serum (Jeil Biotech, Inc., Daegu, South Korea) and 100 units of antibiotic-antimycotic and were cultured at 37 °C in a 10% CO2 incubator.

Cloning of the Mouse Adiponectin Promoter and Construction of a Luciferase Reporter—Mouse genomic DNA was isolated from 3T3-L1 cells with lysis buffer (50 mM Tris, pH 7.5, 50 mM EDTA, 100 mM NaCl, and 2% SDS). Conditions for PCR were as follows: 2 min for 1 cycle (94 °C, 10′ PCR buffer, 5 units of LA (long amplification) Taq polymerase (TaKaRa), in a 50-μl reaction volume. The PCR cycle was: 30 s at 95 °C, followed by 30 cycles of 12 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C, and then 5 min at 72 °C. The primers used were as follows: forward (984 bp), 5′-GGT GCA GTT GGC TGG TAC CCC AGA GCT AAT AGA TAC-3′; reverse (410 bp), 5′-CCT GAA CCA CAC AGC TTC AC-3′; reverse, 5′-TTT TGG TCT CTC GAC ATC TGA TCA TGG TAC AGA-3′; the primers included KpnI (5′-prime) and Xhol (3′-prime) recognition sites. The PCR products were cloned into the vector pBluescript, and subcloned into pGEM easy vector (Promega) and pGL3-basic vector (Promega). Site-directed mutagenesis of the adiponectin promoter (–410 bp) was performed by the QuikChange method (Stratagene) using the following mutagenic primers (mutated sites underlined): mSRE1, 5′-GAG TGQ GAG TAT CAT GGQ CCA ATT AGT GTT GGT GAC TCT CCA GG-3′; mSRE2, 5′-CCA GGA CAA ACT TAT GGG AAA GGG AGG TCT CCG GGC CCC TGA AC-3′. Constructs were confirmed by sequencing.

Transient Transfection and Luciferase Assay—Human embryonic kidney 293 cells or Rat1-IR cells were transfected with DNA constructs 1 day before confluence by the calcium phosphate method as described previously (22). The mammalian expression vector containing amino-terminal ADD1/SREBP1c from amino acids 1 to 403, was derived from pSV-SPORT1 (Invitrogen) as described previously (31). After incubation for 24 h, cell extracts were prepared with lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM CTDA, 10% glycerol, and 1% Triton X-100), and activities of β-galactosidase and luciferase were determined according to the manufacturer’s instructions (Promega). The luciferase activity of each sample, expressed in relative light units, was normalized to its β-galactosidase activity.

ChIP Assay—ChIP assays were performed as described previously (33). In brief, differentiated 3T3-L1 cells were incubated with 200 nM insulin for 48 h. The differentiated adipocytes were cross-linked in 1% formaldehyde for 10 min at 37 °C for 10 min, quenched with glycine, washed twice with PBS containing 1% BSA, and resuspended in 1% NP-40-containing buffer (5 mM PIPES, pH 8.0, 85 mM KCl, and 0.5% Nonidet P-40). Crude nuclei were precipitated and lysed in 200 μl of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), and the nuclear lysates were sonicated and diluted 10-fold with immuno-
Transcriptional Regulation of Mouse Adiponectin Gene

RESULTS

Expression of Adiponectin and ADD1/SREBP1c in Obese and Diabetic Mice—Previous studies have demonstrated that adiponectin expression is significantly reduced in the WAT of several obese and/or diabetic animal models (3, 34). To gain insight into the control of adiponectin gene expression, we investigated the expression of several adipogenic transcription factors that modulate expression of many adipocyte-specific genes in the WAT of investigated the expression of several adipogenic transcription factors in the WAT of obese and/or diabetic animal models (3, 34). To gain insight into the control of adiponectin gene expression, we investigated the expression of several adipogenic transcription factors that modulate expression of many adipocyte-specific genes in the WAT of obese and/or diabetic mouse models.

DNase I footprinting analysis with recombinant ADD1/SREBP1c protein (Fig. 3A). As shown in Fig. 3, two sites, SRE1 and SRE2, were protected from DNase I digestion (lanes 3–5). SRE1 is located between bp –398 and –389 and SRE2 between bp –343 and –334; both SRE motifs from the mouse adiponectin promoter were well conserved with known SREs (Fig. 3B). We also performed gel shift assay with the mouse adiponectin promoter (data not shown).

Binding of ADD1/SREBP1c to the Mouse Adiponectin Promoter—To investigate the possibility that ADD1/SREBP1c is involved in the regulation of adiponectin gene expression, we cloned ~98 kb of the mouse adiponectin promoter from genomic DNA by PCR. Sequence analysis of the promoter revealed several putative binding motifs for transcription factors ADD1/SREBP1c, PPARγ, C/EBPα, and C/EBPβ, and nuclear factor Y (Fig. 2A). Two putative SREs and a putative peroxisomal proliferator-activated receptor regulatory element (PPRE) were present from bp –400 to –330 and at bp –270, respectively. Two C/EBP-binding sites were also located at bp –775 and –264 upstream of the transcription initiation site (Fig. 2A). The promoter also contains several E-box motifs and nuclear factor-Y binding sites, as indicated in Fig. 2A. There is 55.9% sequence identity between the proximal promoter regions of mouse and human adiponectin promoters, and the SREs, PPRE, and C/EBP-binding sites are relatively well conserved (Fig. 2B).

To delineate the precise binding site(s), we performed a DNase I footprinting analysis with recombinant ADD1/SREBP1c protein (Fig. 3A). As shown in Fig. 3, two sites, SRE1 and SRE2, were protected from DNAse I digestion (lanes 3–5). SRE1 is located between bp –398 and –389 and SRE2 between bp –343 and –334; both SRE motifs from the mouse adiponectin promoter were well conserved with known SREs (Fig. 3B). We also performed gel shift assay with the mouse adiponectin promoter (data not shown).

Transactivation of the Mouse Adiponectin Promoter by ADD1/SREBP1c—We examined the ability of ADD1/SREBP1c to transactivate the adiponectin promoter. Two luciferase reporter plasmids were constructed by inserting different 5'-flanking regions (bp –984 and bp –410) of the mouse adiponectin promoter into pGL3 basic vector, yielding constructs pAdiponectin-984-Luc and pAdiponectin-410-Luc, respectively. Coexpression of ADD1/SREBP1c with these reporter constructs led to transcription of the adiponectin promoter (Fig. 4A). To determine which SRE(s) was responsible, we constructed mutant reporters bearing SRE mutations (Fig. 4B). When SRE1 (mSRE1 pAdiponectin-410-Luc) or SRE2 (mSRE2 pAdiponectin-410-Luc) was disrupted, transactivation still occurred. However, when both SRE1 and SRE2 were disrupted (mSRE1 & 2 pAdiponectin-410-Luc), transactivation was no longer observed, implying that either SRE1 or SRE2 is sufficient for activation of the adiponectin promoter by ADD1/SREBP1c (Fig. 4B).

PPARγ and C/EBPα are well known adipogenic transcription factors that modulate expression of many adipocyte-specific genes, and the mouse adiponectin promoter contains a potential PPRE, as well as C/EBP binding sites (Fig. 2). It has been demonstrated recently that PPARγ is involved in transcriptional activation of the human adiponectin promoter. We therefore tested whether PPARγ and C/EBPα are involved in transcriptional activation of the mouse promoter. We performed luciferase
reporter assays in human embryonic kidney 293 cells, which seem to lack most adipogenic transcription factors. As shown in Fig. 4C, coexpression of PPARγ and retinoid X receptor (RXR) α led to transactivation of the mouse promoter, and rosiglitazone, a synthetic PPARγ ligand, further enhanced promoter activity by activating PPARγ (Fig. 4C, lanes 1 and 2). However, transactivation by PPARγ seemed to be less efficient than by ADD1/SREBP1c (Fig. 4C). C/EBPα completely failed to transactivate the mouse adiponectin promoter (Fig. 4D). Therefore, it is likely that adiponectin expression in adipocytes may be regulated by PPARγ as well as by ADD1/SREBP1c.

**Induction of Adiponectin mRNA Expression by ADD1/SREBP1c**—To see whether ADD1/SREBP1c controls adiponectin gene expression in vivo, we overexpressed ADD1/SREBP1c in differentiated 3T3-L1 adipocytes via adenovirus infection. The level of adiponectin mRNA in the infected cells was approximately 2.7-fold higher than in control adipocytes not infected with virus. Adenovirally overexpressed ADD1/SREBP1c also enhanced expression of ADD1/SREBP1c target genes, including FAS and SCD-1 (Fig. 5). Furthermore, expression of
ADD1/SREBP1c itself also increased, presumably via an auto-regulatory loop (35, 36). These results demonstrate that ADD1/SREBP1c is able to promote adiponectin expression in adipocytes, at least at the level of transcription.

**Adiponectin Gene Expression Is Influenced by Nutritional Status and Insulin via ADD1/SREBP1c**—Others and we have demonstrated that expression of ADD1/SREBP1c is tightly regulated by nutritional status (32, 37, 38). Expression of SREBP1 mRNA in WAT was reduced by fasting and induced by feeding or refeeding (Fig. 6A). To determine whether nutrition-dependent expression of ADD1/SREBP1c might affect adiponectin expression, we measured adiponectin mRNA levels upon feeding, fasting, and refeeding. When mice were fasted, adiponectin mRNA expression proved to be low compared with normal feeding or refeeding conditions (Fig. 6A). Thus, adiponectin gene expression is also regulated by nutritional status and is correlated with the regulation of ADD1/SREBP1c.

Insulin positively regulates the expression of ADD1/SREBP1c in fat and liver. To examine the effect of insulin on adiponectin expression, we treated differentiated 3T3-L1 adipocytes with insulin. Expression of adiponectin mRNA, like that of ADD1/SREBP1c, was stimulated by insulin (Fig. 6B). Next, to see whether ADD1/SREBP1c is involved in insulin-dependent adiponectin expression, we performed luciferase reporter assays in the absence and presence of insulin. Mouse adiponectin promoter activity was not significantly changed by insulin in the absence of ADD1/SREBP1c. However, it was strongly stimulated (~2–3-fold) by insulin in the presence of ADD1/SREBP1c (Fig. 6C). To confirm that ADD1/SREBP1c mediates this insulin-dependent adiponectin expression, we performed ChIP assays. Nuclear lysates of differentiated adipocytes incubated in the presence of insulin were immunoprecipitated with antibodies against ADD1/SREBP1c after formaldehyde cross-linking, and association of ADD1/SREBP1c

[Fig. 3. Binding of ADD1/SREBP1c to the mouse adiponectin promoter. A, DNase I footprint of the mouse adiponectin promoter. Lane 1, G/A ladder; lanes 2 and 6, DNase I treatment of the mouse adiponectin promoter in the absence of recombinant ADD1/SREBP1c protein; lanes 3, 4, and 5, in the presence of increasing concentrations of recombinant ADD1/SREBP1c (0, 0.5, 1, or 2 μg). B, sequence comparison of putative SREs in the mouse adiponectin promoter with conserved SRE motifs.]
with the endogenous adiponectin promoter was assessed by PCR amplification of pelleted DNA. Although ADD1/SREBP1c clearly bound to the mouse adiponectin promoter without insulin, its binding was substantially enhanced by insulin treatment, as expected (Fig. 6D).

We also overexpressed ADD1/SREBP1c in differentiated 3T3-L1 adipocytes via adenovirus and examined adiponectin protein levels by Western blot analysis (Fig. 7). Expression of adiponectin protein was about 2.4-fold higher in ADD1/SREBP1c-infected adipocytes than in control, uninfected adipocytes (Fig. 7A, lanes 1 and 2). Insulin treatment also increased the level of adiponectin protein, and ADD1/SREBP1c enhanced this effect (Fig. 7A, lanes 3 and 4). Because adiponectin is secreted by adipocytes, we sought to determine whether
Intensities were determined by imaging of ethidium bromide staining. Products were analyzed by 0.7% agarose gel electrophoresis, and band intensities were determined by imaging of ethidium bromide staining.

ADD1/SREBP1c was overexpressed in differentiated 3T3-L1 adipocytes by adenovirus infection. 3T3-L1 adipocytes were infected with adenovirus containing ADD1/SREBP1c or not, and total RNAs were isolated and analyzed by semi-quantitative RT-PCR. B, quantitation of induced mRNA levels of ADD1/SREBP1c, adiponectin, FAS, and SCD-1 after adenovirus infection. Expression levels are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RT-PCR products. RT-PCR products were analyzed by 0.7% agarose gel electrophoresis, and band intensities were determined by imaging of ethidium bromide staining.

ADD1/SREBP1c affects its secretion; this was indeed the case (Fig. 7B). These results suggest that ADD1/SREBP1c regulates adiponectin expression at the post-transcriptional as well as the transcriptional level.

**DISCUSSION**

Adipose tissue expresses and secretes diverse adipocytokines that affect lipid and glucose metabolism, feeding behavior, energy balance, and insulin sensitivity. In obese subjects, defined as having an accumulation of excess adipose tissue, adipocytes are frequently enlarged so that the secretory profile of the larger adipocytes becomes altered and increasingly dysregulated compared with that of smaller ones from normal subjects. Thus, it has been suggested that defective regulation of adipocytokines is closely related to metabolic disorders in obesity. For example, TNFα has been implicated in insulin resistance (39–41). TNFα mRNA and protein levels are elevated in the adipose tissue of obese and type II diabetic animal models and humans (39, 41, 42). Leptin, another adipokine, suppresses food intake and stimulates energy expenditure, and its expression is increased in obese animals, as is the recently identified resistin (43–47). However, physiological roles of resistin are not clearly understood. Although the levels of most adipocytokines increase in obesity, because of the increase in the total mass of body fat, adiponectin expression is reduced in subjects with obesity, type 2 diabetes or atherosclerosis (3, 7–9, 21, 48). On the other hand, adiponectin administration induces weight loss and improves insulin resistance by increasing fatty acid utilization by activating AMP kinase and inhibiting hepatic glucose production (13–15, 49).

Adiponectin gene expression is regulated by several extracellular signals, including insulin, TNFα, and β-adrenergic agonists, although the link between these signals and adiponectin gene expression, in most cases, remains to be elucidated. For instance, insulin stimulates the expression and secretion of adiponectin in adipocytes, although there are some controversies. Insulin is a key anabolic hormone regulating systemic energy balance by coordinating the storage, mobilization, and utilization of free fatty acids and glucose in liver, skeletal muscle, and adipose tissue. The postprandial rise in plasma insulin increases uptake of glucose and its conversion to glycogen and triglyceride and suppresses glucose production. Therefore, it is of interest to note that insulin stimulates expression of adiponectin and its secretion from adipocytes (Figs. 6 and 7); increased expression of adiponectin is essential to reverse insulin resistance, which occurs when the body fails to respond properly to insulin and is an important factor in the development of type 2 diabetes. Although adiponectin reduces insulin resistance, it does not directly affect insulin secretion or plasma insulin levels (14). Rather, it promotes insulin sensitivity by several mechanisms, including improvement of insulin signaling, increase of fatty acid oxidation, inhibition of gluconeogenesis, and suppression of TNFα signaling (14–16, 22).

ADD1/SREBP1c belongs to the SREBP family of basic helix-loop-helix leucine-zipper transcription factors that play important roles in lipid metabolism (31, 32, 50–52). SREBPs consist of two proteins, SREBP1 and SREBP2 (52, 53). The SREBP1 gene produces two isoforms, SREBP1a and SREBP1c, by use of alternative promoters (52, 54, 55). ADD1/SREBP1c is involved in adipocyte differentiation, insulin sensitivity, and fatty acid metabolism (32, 50, 51). It is highly expressed in adipose tissues and liver, and its transcription is induced at an early stage of adipocyte differentiation, suggesting a role in the induction of the many adipogenic genes that orchestrate adipocyte differentiation and lipid homeostasis (50, 51). In fact, ADD1/SREBP1c stimulates the expression of many genes involved in lipogenesis and adipogenesis including PPARγ, FAS, acetyl CoA carboxylase, lipoprotein lipase, and resistin (32, 33, 50, 56–60).

ADD1/SREBP1c mRNA expression and protein levels are evidently down-regulated in obese and diabetic animal models (Fig. 1). It has been reported that ADD1/SREBP1c expression is decreased in adipose tissue of obese and diabetic humans and that ADD1/SREBP1c expression was lower in obese mice when the genome-wide gene expression profile was investigated (61–63). As described above, mRNA and plasma levels of adiponectin are also reduced in obese subjects (Fig. 1). Therefore, it is likely that common signaling pathways exist to regulate both adiponectin and ADD1/SREBP1c gene expression in adipocytes and that ADD1/SREBP1c is an important transcription factor mediating adiponectin gene expression.

Others and we have shown previously that the expression level of ADD1/SREBP1c is tightly regulated by insulin and nutritional status to coordinate lipid and glucose metabolism in adipose tissue (32). Herein, we have shown that insulin-dependent adiponectin expression is mediated by ADD1/SREBP1c because insulin stimulated transactivation of the adiponectin promoter by ADD1/SREBP1c (Fig. 6). Furthermore, ADD1/SREBP1c enhanced the synthesis and secretion of adiponectin in differentiated adipocytes (Figs. 5 and 7), implying that ADD1/SREBP1c increases insulin sensitivity via its
action on adiponectin. In this regard, it is likely that ADD1/SREBP1c plays a critical role, not only in the insulin-dependent gene expression that coordinates fatty acids and glucose metabolism but also in stimulating adiponectin expression to improve the insulin sensitivity.

TNFα expression is increased in the obese state and has been implicated in obesity-linked insulin resistance (39–42). TNFα stimulates lipolysis, thus increasing plasma free fatty acids and directly interfering with insulin signaling (40, 64). Like adiponectin, ADD1/SREBP1c mRNA expression is markedly
inhibited by TNFα through its action in blocking insulin-dependent activation of ADD1/SREBP1c expression (65). Consequently, it has been suggested that selective down-regulation of ADD1/SREBP1c in the adipose tissue of obese subjects contributes to the partitioning of free fatty acids between insulin-sensitive organs, such as skeletal muscle and liver, where they could impair insulin action via their lipotoxicity (66). These notions may provide a clue about how TNFα could impair insulin action via their lipotoxicity (66).

In summary, we have shown for the first time that ADD1/SREBP1c transactivates the mouse adiponectin promoter and stimulates adiponectin mRNA and protein expression in adipocytes. We have also provided evidence that ADD1/SREBP1c is responsible for insulin-dependent adiponectin gene expression. Because ADD1/SREBP1c is a key transcription factor coordinating lipid and glucose metabolism in response to insulin, it is possible that fine-tuning of ADD1/SREBP1c expression in adipocytes exerts an effect on adiponectin expression. Increased understanding of the mechanisms regulating ADD1/SREBP1c and adiponectin gene expression promises to contribute to the development of approaches to the treatment of obesity and type 2 diabetes.

REFERENCES

1. Saltiel, A. R. (2001) Nat. Med. 7, 887–888
2. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) J. Biol. Chem. 270, 26746–26749
3. Hu, E., Liang, P., and Spiegelman, B. M. (1996) J. Biol. Chem. 271, 10697–10703
4. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsuura, K. (1996) Biochim. Biophys. Res. Commun. 221, 286–289
5. Nakano, Y., Tobe, T., Chou-Mura, N. H., Mazda, T., and Tomita, M. (1996) J. Biochem. (Tokyo) 120, 803–812
6. Saito, K., Tobe, T., Minoshima, S., Asakawa, S., Sumiya, J., Yoda, M., Nakano, Y., Shimizu, N., and Tomita, M. (1999) Gene 229, 67–73
7. Arita, Y., Kimura, S., Hamada, K., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoa, K., Kuriyama, Y., Nishida, M., Yamashita, S., Okubo, K., Matsuzawa, Y., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuura, Y. (1999) Biochim. Biophys. Res. Commun. 257, 79–83
8. Hotta, K., Funahashi, T., Redin, N. L., Oettiker, H. K., Arita, Y., Hansen, B. C., and Matsuzawa, Y. (2001) Diabetes 50, 1128–1133
9. Stefan, N., Bunt, J. C., Salbe, A. D., Funahashi, T., Matsuzawa, Y., and Tataranni, P. A. (2002) J. Clin. Endocrinol. Metab. 87, 4652–4656
10. Lindsay, R. S., Funahashi, T., Hanson, R. L., Matsuzawa, Y., Tanaka, S., Tataranni, P. A., Knowler, W. C., and Krakoff, J. (2002) Lancet 359, 57–58
11. Lindsay, R. S., Funahashi, T., Krakoff, J., Matsuzawa, Y., Tanaka, S., Kokes, S., Bennett, P. H., Tataranni, P. A., Knowler, W. C., and Hanson, R. L. (2003) Diabetes 52, 2419–2425
12. Wu, X., Motoshima, H., Mahadev, K., Stalker, J. T., Scala, R., and Goldstein, B. J. (2005) Diabetes 54, 1355–1363
13. Tomas, E., Tsao, T. S., Saha, A. R., Murrey, E. H., Zhang Cc, C., Itani, S. I., Lodish, H. F., and Ruderman, N. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16309–16313
14. Berg, A. H., Combs, T. P., Xu, D., Brownlee, M., and Scherer, P. E. (2001) Nature 409, 947–953
15. Yamauchi, T., Komoda, K., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akamizu, Y., Fugroel, P., Fouloute, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kanekawa, T. (2002) Nat. Med. 8, 1288–1295
16. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyaama, K., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kishino, S., Chosho, Y., Uchida, K., Hiro, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y. (2002) Nat. Med. 57, 731–737
17. Kadowaki, T., Chiba, T., Moro, M., Matsui, J., Eto, K., Yamashita, T., Komoda, K., Satoh, H., Yano, W., Froguel, P., Nagai, R., Kimura, S., Kadowaki, T., and Noda, T. (2002) J. Biol. Chem. 277, 23883–23886
18. Ouchi, N., Kimura, S., Arita, Y., Nishida, M., Matsuyama, A., Okamoto, Y., Ishigami, M., Kuriyama, H., Kishi, K., Nishizawa, Y., Hotta, K., Mura- guchi, M., Ohmoto, Y., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (2001) Circulation 106, 2473–2476
19. Ouchi, N., Kimura, S., Arita, Y., Nishida, M., Matsuyama, A., Okamoto, Y., Ishigami, M., Kuriyama, H., Kishi, K., Nishizawa, Y., Hotta, K., Mura- guchi, M., Ohmoto, Y., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (2002) J. Clin. Endocrinol. Metab. 87, 4652–4656
20. Tataranni, P. A., Knowler, W. C., and Krakoff, J. (2002) Lancet 359, 57–58
21. Lindsay, R. S., Funahashi, T., Hanson, R. L., Matsuzawa, Y., Tanaka, S., Tataranni, P. A., Knowler, W. C., and Hanson, R. L. (2003) Diabetes 52, 2419–2425
22. Wu, X., Motoshima, H., Mahadev, K., Stalker, J. T., Scala, R., and Goldstein, B. J. (2005) Diabetes 54, 1355–1363
23. Tomas, E., Tsao, T. S., Saha, A. R., Murrey, E. H., Zhang Cc, C., Itani, S. I., Lodish, H. F., and Ruderman, N. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16309–16313
24. Berg, A. H., Combs, T. P., Xu, D., Brownlee, M., and Scherer, P. E. (2001) Nature 409, 947–953
25. Yamauchi, T., Komada, K., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akamizu, Y., Fugroel, P., Fouloute, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kanekawa, T. (2002) Nat. Med. 8, 1288–1295
26. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyaama, K., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kishino, S., Chosho, Y., Uchida, K., Hiro, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y. (2002) Nat. Med. 57, 731–737
27. Kadowaki, T., Chiba, T., Moro, M., Matsui, J., Eto, K., Yamashita, T., Komoda, K., Satoh, H., Yano, W., Froguel, P., Nagai, R., Kimura, S., Kadowaki, T., and Noda, T. (2002) J. Biol. Chem. 277, 23883–23886
