Genes: Structure and Regulation:

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

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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)/sterol regulatory element-binding protein 1c (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.

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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 adipocytokine 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

AMP kinase and ligand-dependent PPARα[1] 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 chromatin immunoprecipitation (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 Schüll). Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (Promega) and [α-^{32}P]dCTP (Amersham Biosciences). cDNAs used as probes were as follows: ADD1/SREBP1c, adiponectin, PPARγ, C/EBPα, fatty acid synthase (FAS) and adipose fatty acid-binding protein.

Western Blot Analysis—Fat tissue was lysed in TGN buffer with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 500 mM NaF, 1 mM Na_3VO_4, 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 with 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 ApaI and EcoRI to obtain 5′- and 3′-overhanging ends. They were labeled with Klenow fragment and [α-^{32}P]dATP, then purified by PAGE. Binding experiments were performed with 50,000 cpm (~1 ng) of probe per reaction in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 7% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly (dI-dC) · poly(dI-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 $MgCl_2$, 5 mM $CaCl_2$ and 7% (v/v) glycerol was added to the reaction, which was then 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% (w/v) polyacrylamide/7 M 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% CO_2 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% CO_2 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% (v/v) bovine calf serum (Jeil Biotech. Inc., Daegu, South Korea) and 100 units of antibiotic-antimycotic and were cultured at 37 °C in a 10% CO_2 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 mM concentrations of each primer, 0.6 mM concentrations of each dNTP, 1× 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′-GTT GCA GTT GGC TGG TAC CCC AGA GCT AAT AAT AGA TAG-3′; forward (−410 bp), 5′-CCT GAA CCA CAC AGC TTC AC-3′; reverse, 5′-TTT TGG TGT CTC GAG ATC CAC TGA CAA TCG TAC AGA CAG-3′. The primers included KpnI (5′-primer) and XhoI (3′- primer) restriction sites. The PCR products were digested with KpnI and XhoI, and subcloned into pGEM easy vector (Promega) and pGL3-basic vector (Promega). Site-directed mutagenesis of the adiponectin promoter (−410 bp)-Luc plasmids was performed with the QuikChange kit (Stratagene) using the following mutagenic primers (mutated sites underlined): mSRE1, 5′-GAG TGG GAG TAT CAT GCG CCA ATT AGT GTT GTT 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 or Rat1-IR cells were transfected with DNA constructs 1 day before confluence by the calcium phosphate method as described previously (31, 32). The mammalian expression vector for ADD1, 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 CDTA, 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 at 37 °C for 10 min and resuspended in 200 μl of Nonidet P-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-

[1] The abbreviations used are: PPAR, peroxisomal-activated receptor; TNFα, tumor necrosis factor α; ADD1, adipocyte determination- and differentiation-dependent factor 1; SREBP1c, sterol regulatory element-binding protein 1c; WAT, white adipose tissue; C/EBP, CCAAT enhancer-binding protein; ChIP, chromatin immunoprecipitation; FAS, fatty acid synthase; DMEM, Dulbecco's modified Eagle's medium; CDTA, 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; RT, reverse transcription; SCD-1, stearoyl-CoA desaturase-1; PPRE, peroxisomal-activated receptor regulatory element; SRE, sterol regulatory element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

precipitation buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton X-100). They were then incubated with Protein A-Sepharose CL-4B (Amersham Biosciences) and anti-ADD1 antibodies for 2 h at 4 °C. After successive washes, immunocomplexes containing DNA were eluted, and the precipitated DNA was amplified by PCR. Conditions for PCR were as follows: 0.25 μM concentrations of each primer, 0.1 mM concentrations of each dNTP, 1× PCR buffer, 1 unit of Ex Taq polymerase (TaKaRa), 0.06 mCi/ml [α-^{32}P]dCTP in a 20-μl reaction volume. The products were resolved on 6% polyacrylamide/1× Tris-borate/EDTA gels. Primers used were as follows: −572 adiponectin-forward (f), 5′-GGT GCT GGG AAT TGA ACT CA-3′; −213 adiponectin-reverse (r), 5′-CCT GTT TCC AGG CTT TGG CC-3′; −349 PPARγ2-f, 5′-CTG TAC AGT TCA CGC CCC TC-3′; −51 PPARγ2-r, 5′-TCA CAC TGG TGT TTT GTC TAT G-3′; GAPDH-f, 5′-GTG TTC CTA CCC CCA ATG TG-3′; GAPDH-r, 5′-CTT GCT CAG TGT CCT TGC TG-3′.

Adenovirus Infection and Semiquantitative RT-PCR—Differentiated 3T3-L1 adipocytes were infected with 1 ml of adenovirus-containing DMEM at a titer of 10 plaque-forming units per cell and incubated for 12 h at 37 °C. The culture medium was then adjusted to 2 ml with DMEM supplemented with 10% fetal calf serum. After incubation for a total of 48 h, cells were harvested for RNA isolation using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RT-PCR reactions were performed with the SuperScript first-strand synthesis system for RT-PCR kit (Invitrogen). ADD1/SREBP1c, adiponectin, FAS, and stearoyl-CoA desaturase-1 (SCD-1) cDNAs were amplified for 30, 28, 29, and 29 (non-saturating) cycles, respectively. RT-PCR products were analyzed by 0.7% agarose gel electrophoresis, and band intensities were compared by imaging ethidium bromide staining (Scion Image; Scion Corporation, Frederick, MD). Primers used were as follows: ADD1/SREBP1c-f, 5′-GGG AAT TCA TGG ATT GCA CAT TTG AA-3′; ADD1/SREBP1c-r, 5′-CCG CTC GAG GTT CCC AGG AAG GGT-3′; adiponectin-f, 5′-ATG CTA CTG TTG CAA GCT CTC-3′; adiponectin-r, 5′-GTT GGT ATC ATG GAA GAG AAG-3′; FAS-f, 5′-TGC TCC CAG CTG CAG GC-3′; FAS-r, 5′-GCC CGG TAG CTC TGG GTG TA-3′; SCD-1-f, 5′-TGG GTT GGC TGC TTG TG-3′; SCD-1-r, 5′-GCG TGG GCA GGA TGA AG-3′; GAPDH-f, 5′-TGC ACC ACC AAC TGC TTA G-3′; GAPDH-r, 5′-GGA TGC AGG GAT GAT GTT C-3′.

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 in the WAT of *ob/ob* and *db/db* mice. As shown in Fig. 1, the expression of PPARγ, C/EBPα, and aP2 was not altered in the WAT of mice. However, the level of ADD1/SREBP1c mRNA was found to be substantially decreased, as was the level of adiponectin mRNA (Fig. 1*A*). ADD1/SREBP1c protein levels were also lower (Fig. 1*B*), indicating that ADD1/SREBP1c may influence adiponectin expression in adipose tissue.

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 ~0.98 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/EBPs, and nuclear factor Y (Fig. 2*A*). 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. 2*A*). The promoter also contains several E-box motifs and nuclear factor-Y binding sites, as indicated in Fig. 2*A*. 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. 2*B*).

To delineate the precise binding site(s), we performed a

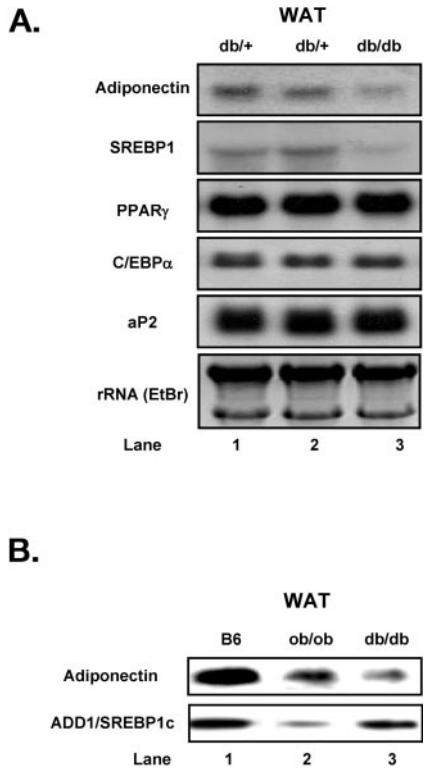

FIG. 1. **mRNA and protein profiles of adiponectin and ADD1/SREBP1c in WAT of C57BL/6, *db*/+, *ob/ob*, and *db/db* mice.** mRNA (*A*) and protein (*B*) levels in the WAT of each type of mouse were examined by Northern or Western blot analysis, respectively. *aP2*, adipose fatty acid-binding protein.

DNase I footprinting analysis with recombinant ADD1/SREBP1c protein (Fig. 3*A*). 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. 3*B*). We also preformed 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. 4*A*). To determine which SRE(s) was responsible, we constructed mutant reporters bearing SRE mutations (Fig. 4*B*). 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. 4*B*).

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 transactivation of the mouse promoter. We performed luciferase

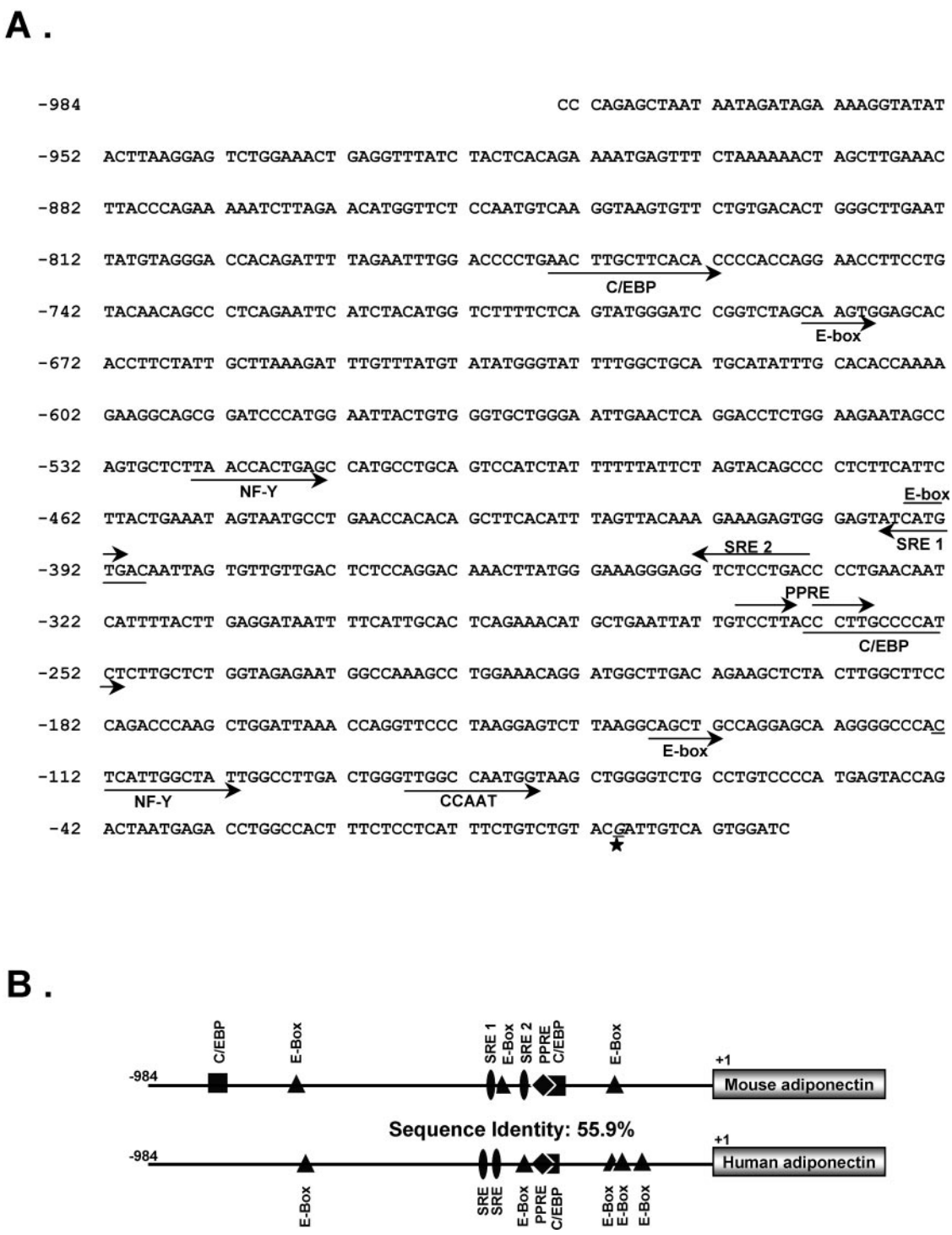

FIG. 2. **Putative binding sites of several transcription factors in the mouse adiponectin promoter (984 bp).** *A*, the sequence of the mouse adiponectin gene is shown and numbered from the transcription initiation site. Two putative SRE motifs, three E-boxes, one PPRE, two C/EBP sites, nuclear factor-Y, and CCAAT are marked with *arrows*. *B*, schematic representation of the mouse and human adiponectin promoters. The putative SREs (*black ovals*), C/EBP-binding sites (*black squares*), E-boxes (*black triangles*), and PPRE (*black diamonds*) are indicated.

reporter assays in human embryonic kidney 293 cells, which seem to lack most adipogenic transcription factors. As shown in Fig. 4*C*, 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. 4*C*, *lanes 1* and *2*). However, transactivation by PPARγ seemed to be less efficient than by ADD1/SREBP1c (Fig. 4*C*). C/EBPα completely failed to transactivate the mouse adiponectin promoter (Fig. 4*D*). 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

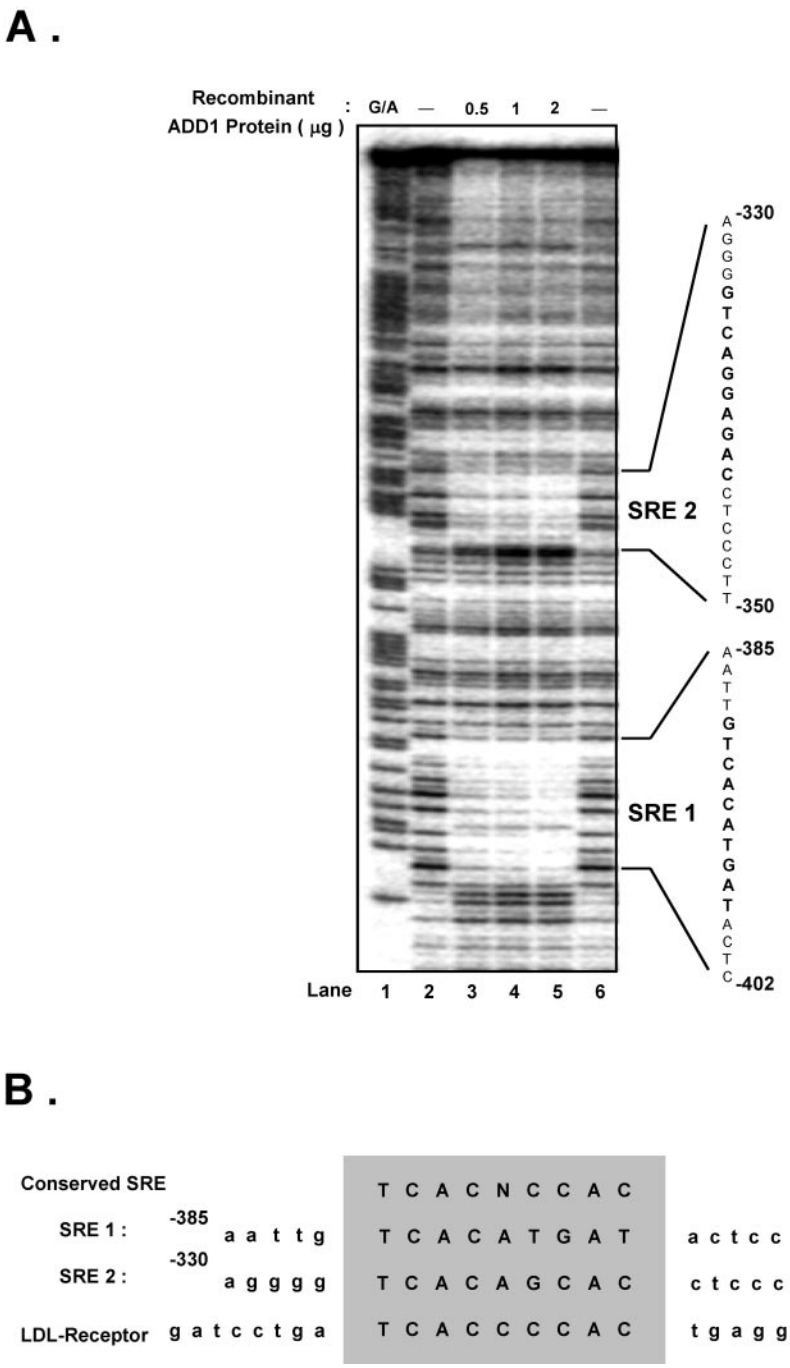

FIG. 3. **Binding of ADD1/SREBP1c to the mouse adiponectin promoter.** *A*, DNase I footprint of the mouse adiponectin promoter. *Lane 1*, G/A ladder; *lanes 2* and *6*, DNase I treatment of the mouse adiponectin promoter in the absence of recombinant ADD1/SREBP1c protein; *lanes 3*, *4*, and *5*, in 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.

ADD1/SRBEP1c itself also increased, presumably via an autoregulatory 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. 6*A*). 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. 6*A*). 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. 6*B*). 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. 6*C*). 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

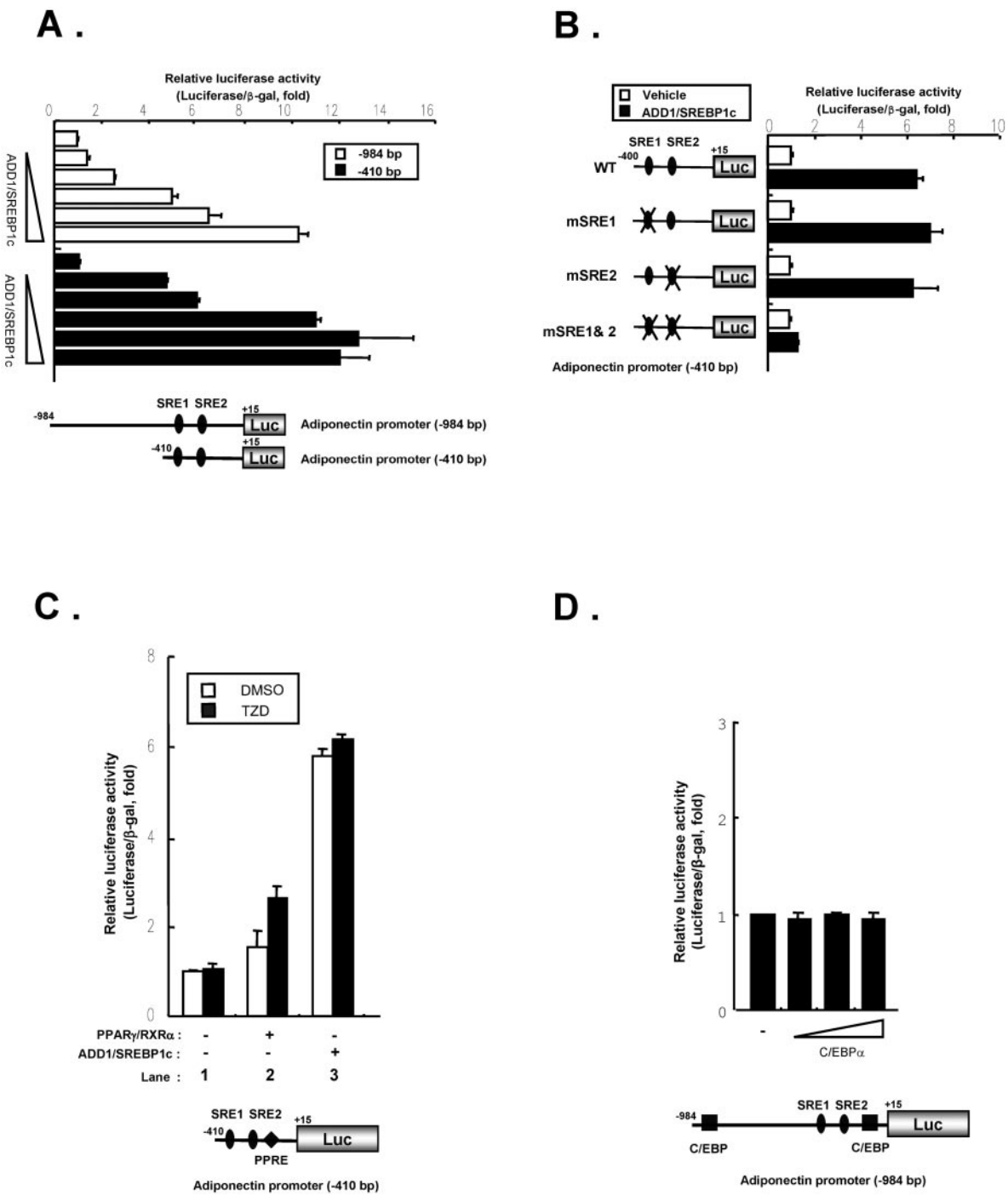

FIG. 4. **Transactivation of adiponectin by ADD1/SREBP1c.** *A*, pAdiponectin-984-Luc or pAdiponectin-410-Luc reporters were cotransfected together with an ADD1/SREBP1c expression vector (ADD1–403) and pCMV-β-gal into human embryonic kidney 293 cells. *B*, the adiponectin promoter activity of ADD1/SREBP1c is dependent on two putative SRE motifs. pAdiponectin-410-Luc or mutant reporter genes were cotransfected together with the ADD1/SREBP1c expression vector and pCMV-β-gal into human embryonic kidney 293 cells. *C*, ADD1/SREBP1c and PPARγ independently transactivate the mouse adiponectin promoter. The pAdiponectin-410-Luc reporter gene was transfected with ADD1 or PPARγ expression vector. After transfection, cells were incubated overnight in serum-free DMEM with 0.5% BSA and treated with or without 1 μM rosiglitazone for 24 h. *DMSO*, dimethyl sulfoxide; *TZD*, thiazolidinedione. *D*, C/EBPα dose not transactivate the mouse adiponectin promoter. The pAdiponectin-984-Luc reporter gene was transferred together with the C/EBPα expression vector and pCMV-β-gal into human embryonic kidney 293 cells. Luciferase activities were normalized to β-galactosidase activity and the values are expressed as fold activity relative to the control. *Error bars* indicate standard deviations ($n = 3$).

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. 6*D*).

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. 7*A*, *lanes 1* and *2*). Insulin treatment also increased the level of adiponectin protein, and ADD1/SREBP1c enhanced this effect (Fig. 7*A*, *lanes 3* and *4*). Because adiponectin is secreted by adipocytes, we sought to determine whether

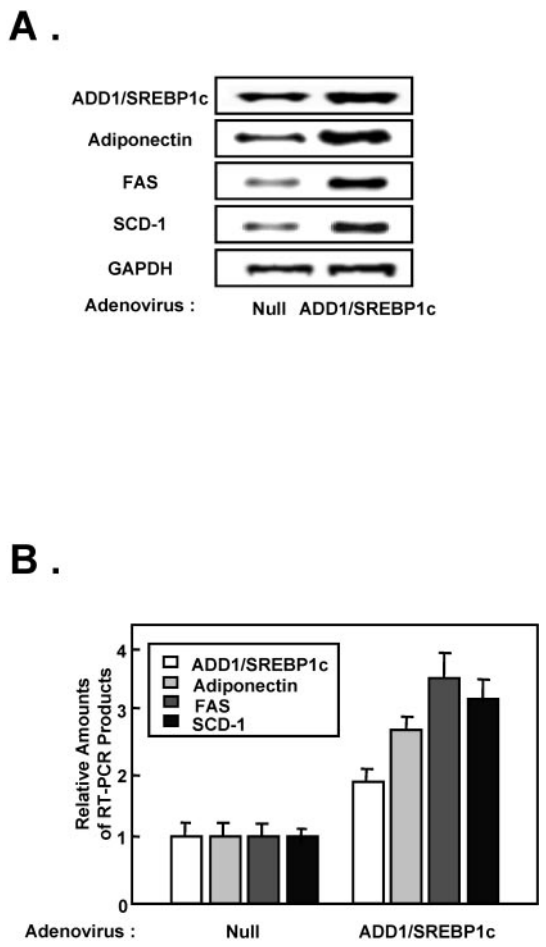

FIG. 5. **Induction of adiponectin mRNA by ADD1/SREBP1c.** *A*, ADD1/SREBP1c was overexpressed in differentiated 3T3-L1 adipocytes by adenovirus infection. 3T3-L1 adipocytes were infected with adenovirus containing ADD1/SREBP1c or not, and total RNAs were isolated and analyzed by semi-quantitative RT-PCR. *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. 7*B*). 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 adipocytokine, 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

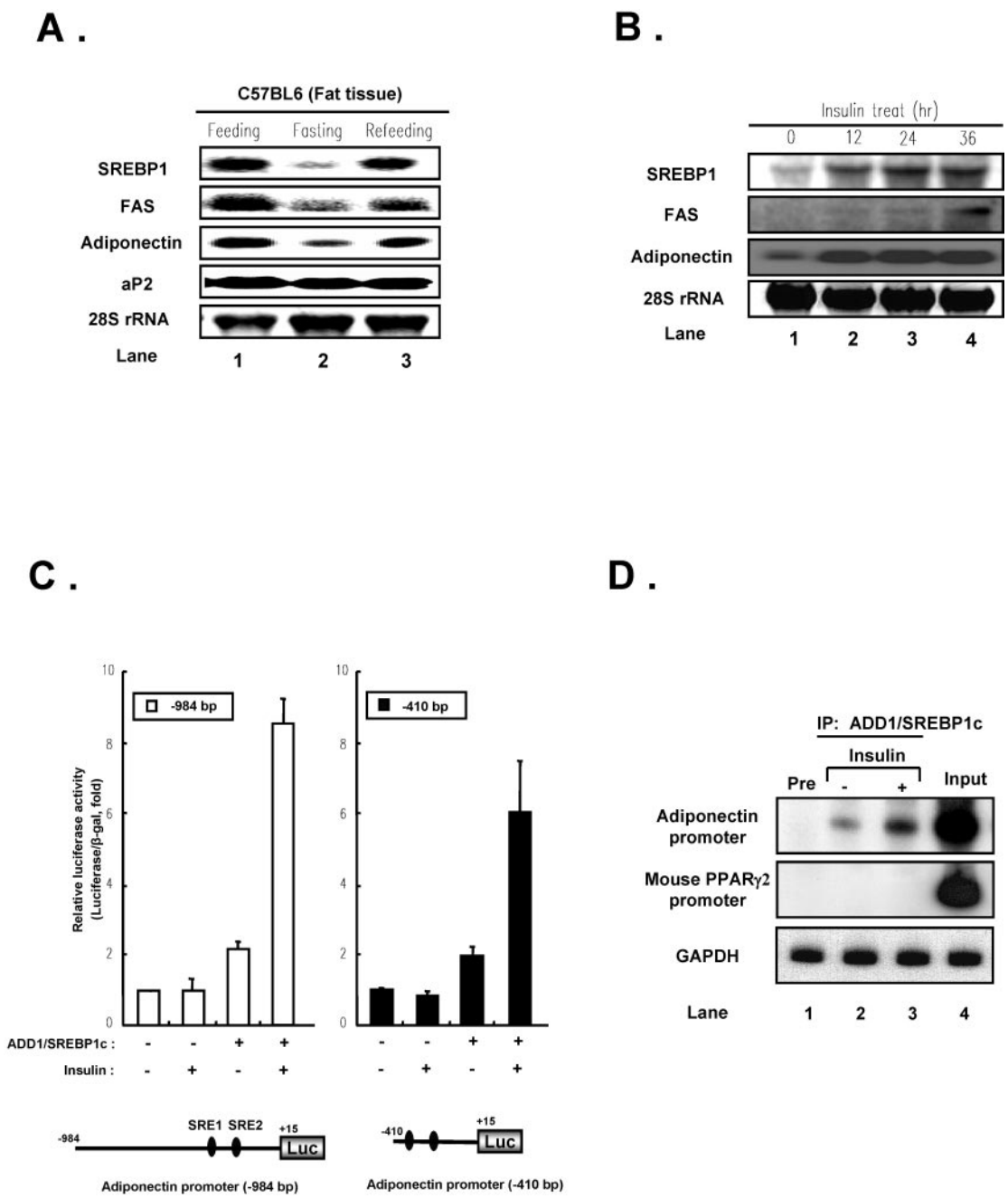

FIG. 6. **Regulation of adiponectin gene expression by nutrient and insulin.** *A*, nutritional regulation of SREBP1, FAS, and adiponectin expression. mRNA expression levels in adipose tissue from fed, fasted, and re-fed mice compared by Northern blot analysis. Mice (male C57BL/6) were sacrificed after nutritional challenge, and total RNA was prepared from abdominal adipose tissue. The mice were divided into three groups. Fed controls were allowed free access to food for 12 h (*lane 1*). The fasted group was denied access to food for 16 h (*lane 2*). The re-fed group was allowed free access to food for 8 h after 16 h of fasting (*lane 3*). The expression levels of feeding, fasting, and refeeding groups were quantified and normalized to 28 S rRNA. *B*, time-dependent effects of insulin on SREBP1, FAS, and adiponectin gene expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated overnight in serum-free low glucose DMEM containing 0.5% BSA and treated with insulin (100 nM). mRNA levels of SREBP1 and adiponectin were examined by Northern blot. 28 S rRNA were used to normalize RNA loading. *C*, transactivation of the mouse adiponectin promoter by ADD1/SREBP1c. Rat1-IR cells were cotransfected with pAdiponectin-984-Luc or pAdiponectin-410-Luc reporter and ADD1/SREBP1c expression vector. After transfection, cells were incubated overnight in serum-free DMEM with 0.5% BSA and treated with (*lanes 2*, *4* and *6*) or without (*lanes 1*, *3* and *5*) 100 nM insulin for 24 h. They were harvested and assayed for luciferase and β-galactosidase activity. *D*, induction of ADD1/SREBP1c binding to the mouse adiponectin promoter with insulin. ChIP assay of the mouse adiponectin promoter. Fully differentiated 3T3 L1 cells were cultured in the presence or absence of insulin. They were cross-linked and immunoprecipitated with polyclonal anti-ADD1/SREBP1c antibodies (*lanes 2* and *3*), and the immunoprecipitated DNA fragments were amplified by PCR. *Lane 4* shows the amplified adiponectin promoter, PPARγ2 promoter, and *GAPDH* from 1% of input DNA. GAPDH fragments were amplified to normalize input DNA.

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

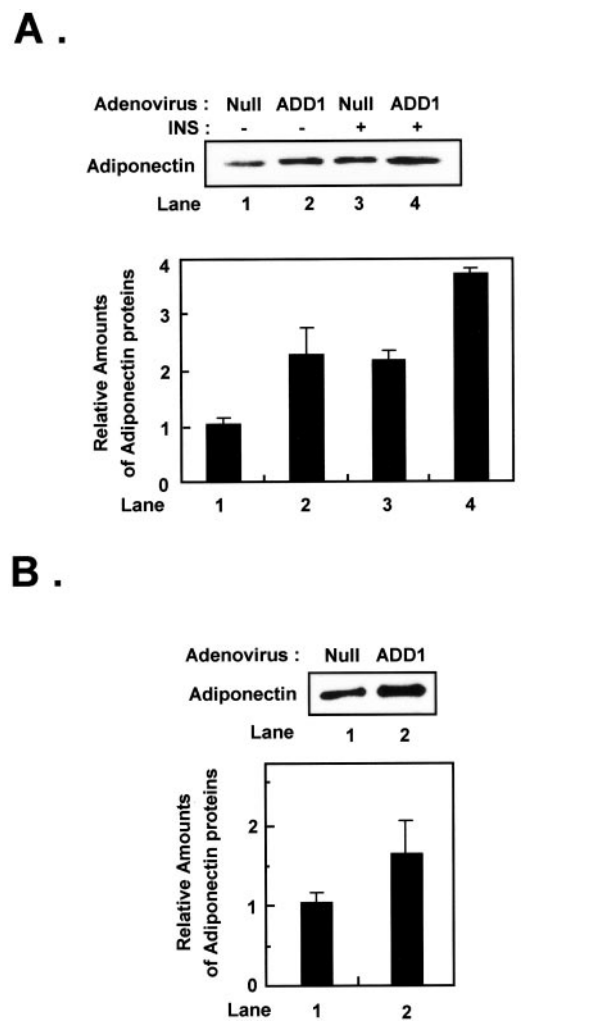

FIG. 7. **Induction of adiponectin protein synthesis and secretion by insulin via ADD1/SREBP1c.** *A*, ADD1/SREBP1c was overexpressed in differentiated 3T3-L1 adipocytes by adenovirus infection. After insulin treatment, 3T3-L1 adipocytes containing no virus (*Null*) or ADD1/SREBP1c virus were harvested, and total protein was isolated and analyzed by Western blot. *B*, culture medium from 3T3-L1 adipocytes infected or not with ADD1/SREBP1c was analyzed by Western blot. Protein synthesis and secretion levels were normalized to those of adipocytes not infected with adenovirus (*Null*). Each experiment was repeated independently at least three times.

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 is able to decrease adiponectin gene expression in adipocytes, because TNFα reduces ADD1/SREBP1c expression. Of course, the regulatory networks connecting extracellular signals to ADD1/SREPB1c and adiponectin expression remain to be elucidated. Taken together, these findings show that ADD/SREBP1c is an important transcription factor mediating adiponectin regulation by insulin and TNFα in adipocytes. They also account for why adiponectin gene expression is decreased in insulin-resistant obese and diabetic subjects.

Rosiglitazone, a member of thiazolidinediones, enhances the expression and secretion of adiponectin in *db/db* mice fed a high fat diet as well as in wild-type mice and cultured 3T3-L1 adipocytes (25, 67). Transcriptional activation of PPARγ is stimulated by its ligands, thiazolidinediones, and increases insulin sensitivity by reducing TNFα and increasing adiponectin expression (25). Iwaki *et al.* recently identified a functional PPRE in the human adiponectin promoter that mediates activated PPARγ-dependent adiponectin gene expression (30). We have also mapped a similar PPRE that can be transactivated by PPARγ, in the mouse adiponectin promoter. Although we found several putative binding sites for C/EBPs, we failed to observe C/EBPα-induced activation of the adiponectin promoter. This finding is supported by the observation that adiponectin expression in C/EBPα-deficient cell lines continues to be activated by PPARγ (68). Of course, we cannot rule out the possibility that other C/EBP isoforms such as C/EBPβ or -δ contribute to adiponectin gene expression; indeed, it is possible that both PPARγ and ADD1/SREBP1c regulate adiponectin gene expression in adipocytes to increase insulin sensitivity.

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.

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