Regulation of GLUT4 gene expression by SREBP-1c in adipocytes

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Expression of the GLUT4 (glucose transporter type 4 isoform) gene in adipocytes is subject to hormonal or metabolic control. In the present study, we have characterized an adipose tissue transcription factor that is influenced by fasting/refeeding regimens and insulin. Northern blotting showed that refeeding increased GLUT4 mRNA levels for 24 h in adipose tissue. Consistent with an increased GLUT4 gene expression, the mRNA levels of SREBP (sterol-regulatory-element-binding protein)-1c in adipose tissue were also increased by refeeding. In streptozotocin-induced diabetic rats, insulin treatment increased the mRNA levels of GLUT4 in adipose tissue. Serial deletion, luciferase reporter assays and electrophoretic mobility-shift assay studies indicated that the putative sterol response element is located in the region between bases −109 and −100 of the human GLUT4 promoter. Transduction of the SREBP-1c dominant negative form to differentiated 3T3-L1 adipocytes caused a reduction in the mRNA levels of GLUT4, suggesting that SREBP-1c mediates the transcription of GLUT4. *In vivo* chromatin immunoprecipitation revealed that refeeding increased the binding of SREBP-1 to the putative sterol-response element in the GLUT4. Furthermore, treating streptozotocin-induced diabetic rats with insulin restored SREBP-1 binding. In addition, we have identified an Sp1 binding site adjacent to the functional sterol-response element in the GLUT4 promoter. The Sp1 site appears to play an additive role in SREBP-1c mediated GLUT4 gene upregulation. These results suggest that upregulation of GLUT4 gene transcription might be directly mediated by SREBP-1c in adipose tissue.

Key words: adipocytes, insulin, sterol-response element, sterol-regulatory-element-binding protein-1c (SREBP-1c), type 4 glucose transporter isoform (GLUT4).

INTRODUCTION

GLUT4 (the glucose transporter type 4 isoform) is a member of the glucose transporter family that is mainly expressed in skeletal muscle, heart and adipose tissues. GLUT4 plays a critical role in insulin-stimulated glucose transport in these tissues, with glucose uptake occurring when insulin stimulates the translocation of GLUT4 from the intracellular pool to the plasma membrane [1]. Additionally, the intrinsic activity of GLUT4 can also be regulated by covalent modification [2].

T2DM (type 2 diabetes mellitus) is characterized by defects in both insulin action and insulin secretion [3]. Central to this pathology is insulin resistance, manifested by decreased glucose disposal in response to insulin [4,5]. Studies have reported that T2DM patients display a marked decrease in insulin-stimulated glucose disposal in muscle [6]. One of the mechanisms underlying this defect involves defects in insulin signalling pathway components, including phosphatidylinositol 3-kinase [7], insulin receptor substrate-1 [8,9] and Akt kinase [9–11]. In this diabetic condition, overexpression of the GLUT4 gene in muscle or adipose tissue enhances insulin responsiveness and peripheral glucose utilization [12]. Moreover, transgenic *db*/*db* mice overexpressing the GLUT4 gene showed improved insulin resistance [13]. Regulation of the level of GLUT4 protein is accomplished by increased biosynthesis, decreased degradation or both [14,15]. However the molecular mechanisms that govern regulation of GLUT4 gene expression remain largely unknown.

GLUT4 gene expression is known to be regulated by various physiological conditions. In obese or T2DM human subjects [16,17], GLUT4 gene expression is decreased due to the compensatory hyperinsulinaemia that occurs in these patients [18]. In addition, insulin treatment of 3T3-L1 adipocytes results in a rapid (within 4 h) decrease in GLUT4 mRNA levels [19], suggesting that insulin acts as a negative regulator of GLUT4. In contrast, GLUT4 gene expression is down-regulated in STZ-(streptozotocin)-induced diabetes and chronic fasting, a state of relative insulin deficiency, suggesting that insulin acts as a positive regulator of GLUT4 gene expression [20]. Some studies indicate that during insulin deficiency states, increased intracellular cAMP levels or other transcription factors signal the repression of GLUT4 gene expression [21,22].

ADD1/SREBP-1c (adipocyte determination and differentiation dependent factor 1/sterol-regulatory-element-binding protein-1c) is a *trans*-acting factor that regulates transcription of genes involved in cholesterol and fatty acid synthesis [23–26] and is abundantly expressed in the liver and adipose tissue [27]. In the liver, SREBP-1c has a well documented role as an insulin-mediated transcriptional activator of genes involved in carbohydrate and lipid metabolism [28,29]. Despite the established role of SREBP-1c in the liver, the influence of SREBP-1c on GLUT4 and other genes involved in carbohydrate metabolism in adipocytes is not clear.

In the present study, we identified the SRE (sterol-reponse element) in the promoter region of GLUT4. We also demonstrated

Abbreviations used: ADDN, dominant negative mutant of SREBP-1c; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; FAS, fatty acid synthase; FCS, fetal calf serum; GFP, green fluorescent protein; GLUT4, type 4 glucose transporter isoform; LDLR, low-density lipoprotein receptor; MOI, multiplicity of infection; SCD1, stearoyl CoA desaturase-1; SRE, sterol-response element; SREBP-1, sterol-regulatory-element-binding protein-1; STZ, streptozotocin; T2DM, type 2 diabetes mellitus; WAT, white adipose tissue.

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that SREBP-1c directly mediates the upregulation of GLUT4 in the adipose tissues of the fasting/refeeding cycle or in STZ-treated rats that have been treated with insulin.

EXPERIMENTAL

Animals, diet and the induction of diabetes by STZ

Male Sprague–Dawley rats (weighing approx. 200 g each) were fasted for 48 h and then fed a fat-free, high carbohydrate diet for the indicated period. The fat-free, high carbohydrate diet contained 82 % (w/w) carbohydrates (74 % starch and 8 % sucrose), 18 % (w/w) casein, 1 % vitamin mix and 4 % (w/w) mineral mix. Animals had free access to water at all times. All the materials for the diet were purchased from Harlem Teklad Co. (Madison, WI, U.S.A.). Diabetes was induced in the rats by a single intravenous injection of 0.5 ml 50 mM sodium citrate solution (pH 4.5) containing STZ (50 mg/kg body weight, Sigma). Control rats were injected with 50 mM sodium citrate solution (pH 4.5). The induction of diabetes was monitored by measuring blood glucose level (> 300 mg/dl). The animals were then fed a chow diet for 12 h, after which insulin was administered to the STZ + insulin group. The animals received 0.5 mM/kg insulin (Sigma, Cat. I-9278) intraperitoneally. The control STZ group rats were injected with the same volume of PBS. After the injection of insulin or PBS, the animals were fed a chow diet for 6 h and then killed by halothane anaesthesia.

Cell culture

3T3-L1 preadipocytes (ATCC number CL-173) were cultured in DMEM (Dulbecco's modified Eagle's medium) and supplemented with 10 % (v/v) FCS (fetal calf serum), 100 units/ml penicillin and 100 μg/ml streptomycin. 3T3-L1 preadipocytes were differentiated into adipocytes as described previously [30].

Nuclear lysate preparation and Western blot for mature SREBP-1c detection

Rat epididymal adipose tissues were homogenized for 15–30 sec using a Polytron homogenizer (Wheaton) in 1.5 ml homogenization buffer [20 mM Tris/HCl (pH 7.4), 2 mM $MgCl_2$, 0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, proteases (1 M dithiothreitol and 0.1 M PMSF) and 1 × protease inhibitor cocktail (5 mM leupeptin, 5 mM pepstatin, 5 mM chymostatin and 5 mM aprotinin)]. The homogenates were kept on ice for 10 min and spun down for 5 min at 2500 ***g*** in an Eppendorf 5810R tabletop centrifuge at 4 °C. The pellet was resuspended in 1 ml homogenization buffer including protease inhibitor cocktail and nuclei were spun down at 1000 ***g*** for 5 min at 4 °C. The pellet was resuspended with 300 μl buffer C [20 mM Hepes (pH 7.6), 2.5 % glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail]. Western blot analysis was performed as described previously [31]. Seventy micrograms of nuclear lysate was prepared by adding 1 × SDS sample loading buffer followed by 20 bursts of sonication (Cycle, 0.5; amplitude 80 %) and boiling at 95 °C for 5 min. The protein samples were separated by SDS/PAGE (10 % gel) and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience GmbH). The blotted membranes were immunostained with antibodies for SREBP-1 (1:2000) which was prepared as described previously [25] and α-tubulin (1:4000; Calbiochem, Darmstadt, Germany) and subsequently detected by ECL® (Amersham Biosciences).

Isolation of total RNA and Northern blot analysis

Total cellular RNAs were extracted from the epididymal WAT (white adipose tissue) of Sprague–Dawley rats using TRIzol® reagent (Invitrogen) and were prepared according to the manufacturer's protocol. Labelling of each cDNA probe (SREBP-1c and GLUT4) with [α-^{32}P]dCTP was performed using the Rediprime labelling kit (Amersham Biosciences).

Construction of human GLUT4 promoter–luciferase plasmids

The human GLUT4 promoter fragments spanning −2031/+61 were subcloned into the KpnI and XhoI sites of the pGLM plasmid which was mutated to the E-box upstream of the multiple cloning site of the pGL3 basic vector (Promega) in order to abolish the effect of SREBP-1c. Serial deletions from the 5′ end of human GLUT4 promoter reporter constructs, pHGT4-828, pHGT4-313, pHGT4-119, pHGT4-92, and pHGT4-41 were formed by amplifying the regions of −828/+61, −313/+61, −119/+61, −92/+61 and −41/+61 respectively, and these were subcloned into the pGLM basic vector. The SRE-truncated promoter reporter pHGT4t-133/−36 was constructed from pHGT4d-313 using an SRE-truncated primer (sense: 5′-CCAAACTCTAAACGCTTCTCGCGTCTTTTCCC-3′, anti-sense: 5′-GACGCGAGAAGCCGTTTAGAGTTTGGCTGGAGT-3′). Mutant constructs pHGT4-Sp1m (Ma), pHGT4-SREm (Mb), and pHGT4-Sp1m/SREm (Mab) were generated from pHGT4d-313 with the mutagenic oligonucleotides (30 mer, see Figure 5A) using *Pfu* polymerase (Stratagene). The sequences of all constructs were confirmed using the T7 sequencing kit (Amersham Biosciences). All transfection plasmids were prepared with the Plasmid Midi Kit (Qiagen).

Transient transfection assay

3T3-L1 preadipocytes were plated at a density of 2×10^5 cells/35 mm dish. On the following day, transfection was performed with 0.5 μg of the indicated GLUT4 promoter–luciferase constructs, 0.1 μg of pCMV-β-galactosidase plasmid (Clontech), the indicated amounts of pcDNA3, pCMV7-SREBP-1c and ADDN (the dominant negative mutant of SREBP-1c) expression plasmids [32]. Transient transfections were performed using Lipofectamine Plus reagent (Life Technologies) for 3 h according to the manufacturer's instructions. After two days, cells were washed with PBS and lysed in 150 μl of reporter lysis buffer (Promega). Luciferase activities were measured using the Luciferase Assay System (Promega) and normalized with 20 μg of protein, as measured by the Bradford assay [33].

Electrophoretic mobility-shift assay (EMSA)

Probes for the gel shift assays were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labelled double-stranded oligonucleotides were prepared by mixing a 5-fold molar excess of the complementary single-stranded DNA in 50 mM NaCl which was heated to 90 °C for 5 min and cooled to room temperature (25 °C). Recombinant SREBP-1 and antiserum against SREBP-1 were prepared as described previously [25]. The recombinant Sp1 zinc finger DNA binding domain was prepared as described previously [34]. The labelled probe (50 000 cpm) and purified SREBP-1 and Sp1 [in 25 mM Tris/HCl (pH 7.4), 80 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 10 % (v/v) glycerol] were incubated for 30 min on ice and resolved on a non-denatured 4 % acrylamide gel [acrylamide/bisacrylamide, 29:1 (w/w)] in 1 × TBE [45 mM Tris borate, 0.1 mM EDTA (pH 8.0)] at 4 °C. A competition experiment was performed using a molar excess of unlabelled oligonucleotide including the SRE region

Table 1 Sequence of oligonucleotides used for the EMSA

Mutated bases are represented in bold and underlined.

Oligonucleotide		Sequence (5′→3′)
SREa	sense	GAGGGGGCGTGGCCTTCTGGGGTGTGCGGGCTCCTGGCCAATGGGTGC
	antisense	GCACCCATTGGCCAGGAGCCCGCACACCCCAGAAGGCCACGCCCCCTC
SREb	sense	GAGGGG**TTT**TGGCCTTCTGGGGTGTGCGGGCTCCTGGCCAATGGGTGC
	antisense	GCACCCATTGGCCAGGAGCCCGCACACCCCAGAAGGCCA**AAA**CCCCTC
SREc	sense	GAGGGGGCGTGGCCTTC**AA**GGG**AA**TGCGGGCTCCTGGCCAATGGGTGC
	antisense	GCACCCATTGGCCAGGAGCCCGCA**TT**CCC**TT**GAAGGCCACGCCCCCTC
LDLR	sense	TTGAAAATCACCCCATTGCAGACTCCTCCCCGGC
	antisense	GCCGGGGAGGAGTCTGCAATGGGGTGATTTTCAA

in the low-density lipoprotein receptor (LDLR) promoter [35]. The non-specific competitor, 1.5 μg of poly(dI-dC), was added to each binding reaction. The dried gels were exposed to X-ray film at −70 °C with an intensifying screen. The oligonucleotide sequences used for the EMSA are summarized in Table 1. Mutated bases are represented in bold and are underlined.

Adenovirus preparation and infection

Recombinant GFP (green fluorescent protein), full-length SREBP-1c and ADDN adenoviruses were generated as described previously [29]. Differentiated 3T3-L1 adipocytes were treated with 100 nM insulin and transduced with 5 ml of adenovirus-containing DMEM at the indicated MOI (multiplicity of infection) for 16 h at 37 °C. The culture medium was then adjusted to 10 ml with DMEM supplemented with 10 % FCS. After incubation for a total of 48 h, cells were harvested and RNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

Total RNA was isolated from differentiated 3T3-L1 adipocytes using TRIzol® reagent. For reverse transcription, cDNA was prepared as described previously [36]. Primers for GLUT4 and α-tubulin were designed using MacVector software (Accelrys). The PCR reaction mixture was processed as described previously [36]. Briefly, we used 1 μl of the commercial, ready-to-use mixture LightCycler-DNA Fast Start master hybridization probes (Roche Diagnostics), 5 mM $MgCl_2$ (final concentration) and primers and probes at final concentrations of 5 and 1 μM respectively. Real-time PCR was performed using a LightCycler instrument (Roche Diagnostics). The conditions for thermal cycling were as follows: initial denaturation for 10 min, followed by 40 amplification cycles at 95 °C for 10 s, 55 °C for 5 s and 72 °C for 14 s. Fluorescence was measured at 640 nm (F2 channel) at the end of each annealing phase. The amplification was followed by a melting programme, which started at 45 °C for 15 s and then increased to 95 °C at 0.1 °C/s with the fluorescence signal continuously monitored. The primers and probes used were: GLUT4 sense: 5′-AGAGTCTAAAGCGCCT-3′, antisense: 5′-CCGAGACCAACGTGAA-3′, FAS (fatty acid synthase) sense: 5′-GAAGCTCGTGTTGACTTCTC-3′, antisense: 5′-AGAAGACCACAAAGTAGTCCAG-3′, α-tubulin sense: 5′-CTCGCATCCACTTCCCT C-3′, antisense: 5′-ATGCCCTCACCCACGTAC-3′.

Reverse transcriptase (RT)-PCR

For RT-PCR, RNA was isolated from differentiated 3T3-L1 adipocytes using TRIzol® reagent. For reverse transcription, cDNA was prepared as described previously [36]. RT-PCR was performed as described previously [30]. The conditions for thermal cycling were as follows: initial denaturation for 10 min, followed by 25 amplification cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The sequences used for RT-PCR were as follows: FAS sense: 5′-GAAGCTCGTGTTGACTTCTC-3′, antisense: 5′-AGAAGACCAC AAAGTAGTCCAG-3′, SCD1 (stearoyl CoA desaturase-1) sense: 5′-ATGCCGGCCCACA TGCTCCA-3′, antisense: 5′-CATGAGGATGATGTTCCTCC-3′. β-actin sense: 5′-TTGTAAC CAACTGGGACGATATGG-3′, antisense: 5′-CGACCAGAGGC ATACAGGGACAAC-3′.

ChIP (chromatin immunoprecipitation) assay

The ChIP assay was performed following a modified protocol based on a previously described method [37]. Briefly, rats were killed and perfused with 100 ml of ice-cold PBS (pH 8.0) through the left ventricle of the heart and fixed with 200 ml of 5 % formaldehyde in ice-cold PBS (pH 8.0). Again, the rats were perfused with 100 ml of ice-cold PBS (pH 8.0) to wash out the formaldehyde. Epididymal WAT was homogenized and sonicated. The sonicated supernatant was divided into aliquots (100 μl) for subsequent 10-fold dilution in ChIP dilution buffer [0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl (pH 8.0) and 167 mM NaCl] and precleared with 50 % slurry of protein A–agarose/salmon sperm DNA for 1 h. The antibody was added and incubated overnight at 4 °C. The complexes were collected with 50 % slurry of protein A–agarose/salmon sperm DNA for 1 h. The beads were washed and chromatin complexes were eluted from the beads. The DNA was purified after the cross-linking was reversed and 3 μl of input control or ChIP samples were used as templates for PCR. The primer sets for SRE were used for the GLUT4 promoter. The GLUT4 promoter primers were as follows: sense, 5′-CTTTTGTTCCAGGGACTCTTTT-3′; antisense, 5′-CACAAGCCAAGGATCCGGAGAG-3′.

Statistical analysis

All of the transfection studies were performed in 3–5 separate experiments, where triplicate dishes were transfected. The results are presented as means ± S.D. Statistical analysis was performed using Microsoft Excel. An unpaired t test with a P value of < 0.05 was used to determine statistical significance.

RESULTS

Effect of insulin and refeeding on SREBP-1 and GLUT4 mRNA levels in epididymal WAT

STZ treatment or 48 h fasting resulted in the decrease in GLUT4 mRNA levels in epididymal WAT. Insulin administration restored

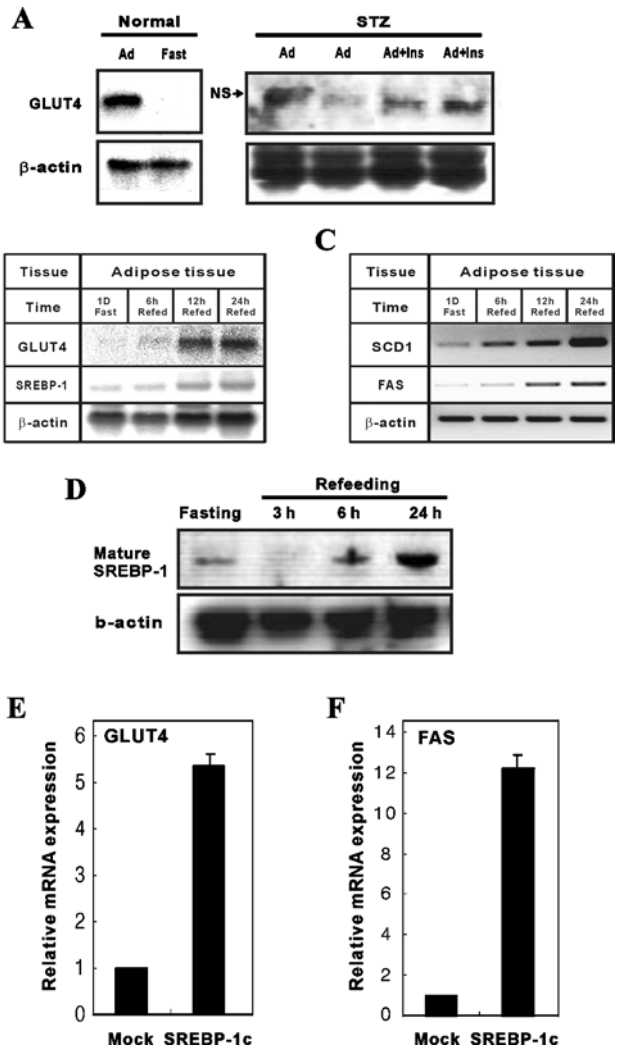

Figure 1 Effect of refeeding on SREBP-1 and GLUT4 mRNA levels in epididymal WAT

(**A**) GLUT4 mRNA levels. Diabetes was induced by administering STZ (50 mg/kg) to Sprague–Dawley rats (approx. 200 g body weight) and the blood glucose concentration was checked to confirm the onset of diabetes. Normal rats were subjected to fasting for 48 h. Insulin (0.5 mM/kg) was administered to STZ-diabetic rats. (**B**) Time course measurements of GLUT4 mRNA levels. Rats were fasted for 48 h and refed a fat-free, high-carbohydrate diet *ad libitum*. After refeeding, the rats were sacrificed at the time periods indicated. Total RNA (20 μg) isolated from epididymal WAT was subjected to 0.9% formaldehyde–agarose gel electrophoresis. The RNA in the gel was transferred to a nylon membrane and hybridized to ^{32}P-labeled cDNAs for GLUT4, SREBP-1 or β-actin. Representative Northern blots are shown for GLUT4, SREBP-1 and β-actin mRNA from normal ($n = 3$) or diabetic ($n = 3$) rats. (**C**) Kinetics of SCD1 and FAS mRNA induction after refeeding. (**D**) Time course measurement of mature SREBP-1 protein expression after refeeding. The mature form of SREBP-1 was measured by fractionating nuclear lysate from adipose tissue as described in the Experimental section. The expression level of endogenous GLUT4 gene (**E**) and FAS (**F**) induced by SREBP-1c adenovirus in 3T3-L1 adipocytes. After infecting SREBP-1c adenovirus at 100 MOI for 48 h in differentiated 3T3-L1 adipocytes, GLUT4 and FAS gene expression was confirmed by real-time PCR. Values are mean $\pm$ S.E.M ($n = 3$). $P < 0.001$ compared to mock group. Ad: *ad libitum*, Ins: insulin treated, NS: non-specific band.

GLUT4 mRNA levels in STZ-induced diabetic rats (Figure 1A), suggesting that insulin promotes expression of the GLUT4 gene. Because SREBP-1c is known to be one of the key mediators of insulin action, we measured both SREBP-1c and GLUT4 mRNA levels in the epididymal WAT of refed rats (Figure 1B). Time course measurement of SREBP-1 mRNA levels in epididymal WAT showed that mRNA levels increased after 6 h of refeeding. SCD1 mRNA was induced after 6 h whereas FAS mRNA was increased after 12 h by refeeding (Figure 1C). In the nuclear fraction of WAT, mature SREBP-1c began to be increased after refeeding for 6 h (Figure 1D). In the differentiated 3T3-L1 adipocytes, the expression level of the endogenous GLUT4 gene was activated by overexpressed adenovirus expressing SREBP-1c (Figure 1E). The FAS gene is known to be regulated by SREBP-1c and was therefore used as a positive control. mRNA levels of the FAS gene were also increased by exogenous SREBP-1c (Figure 1F). These results suggest that GLUT4 expression may be linked to SREBP-1 expression in adipose tissue.

Localization of SRE in the human GLUT4 promoter

A homology search of the consensus sequence between the human and rat GLUT4 promoter regions suggested that highly conserved Sp1 and SRE sites might be present in the region between -125 bp and -77 bp (Figure 2A). To exclude the effect of endogenous GLUT4 and SREBP-1c, 3T3-L1 preadipocytes were used in the transient transfection assay. Promoter reporter (pHGT4-2031) activity was increased by the ectopic expression of SREBP-1c in 3T3-L1 preadipocytes (Figure 2B, $^{*}P < 0.001$, untransfected versus SREBP-1c group). The stimulatory effect of SREBP-1c on the promoter reporter was suppressed by the co-transfection of the ADDN with the SREBP-1c expression vector (Figure 2B, $^{\#}P < 0.05$, SREBP-1c versus SREBP-1c + ADDN group), indicating that SREBP-1c acts as an activating *trans*-acting factor of the GLUT4 promoter.

To localize the putative SRE in the human GLUT4 promoter, serial deletion constructs of the 5′-flanking region of the GLUT4 promoter (phGT4 $-2031/+66$) were prepared and we analysed their responsiveness to SREBP-1c in 3T3-L1 preadipocytes (Figure 2C). The effect of SREBP-1c was decreased by deleting the bases down to -92 bp, indicating that a putative SRE could be located between the -119 bp and -92 bp regions (Figure 2C, $^{*}P < 0.005$, pHGT4d-119 versus pHGT4d-92). Internal deletion of the putative SRE region (pHGT4t-133/36) resulted in a loss of responsiveness to SREBP-1c (Figure 2C).

Identification of an SRE and binding of SREBP-1c in the GLUT4 promoter

EMSA was performed to identify the SRE in the -125 bp and -79 bp region. Recombinant SREBP-1 [25] bound well to the probe (-125 bp/-79 bp fragment) in a dose-dependent manner up to a concentration of 80 ng (Figure 3). The protein–DNA complex formation was abolished by adding 10-fold excess of non-radioactive SRE from the LDLR promoter. The addition of anti-SREBP-1 antibody caused a supershift of the SREBP-1 protein–DNA complex.

Binding of Sp1 and SREBP-1 to the GLUT4 promoter

Figure 4(A) shows the putative *cis*-elements, Sp1 and SREBP-1, binding to the GLUT4 promoter. Combinations of *cis*-element mutants were generated by site-directed mutagenesis in order to confirm the identity of the *cis*-element. SREa, which contains putative Sp1 and SRE binding sites binds both Sp1 and SREBP-1. Binding of SREBP-1 to SREa was confirmed by supershift assay (Figure 4B). The SREb probe, which carries a mutated Sp1 binding site, forms a protein–DNA complex when recombinant SREBP-1 is added. SREb failed to bind Sp1 (Figure 4C). Introduction of a mutation into the core sequence of SRE (Figure 4D) caused a loss of SREBP-1 binding. These data indicate that the Sp1 and SRE binding sites are located at -122 bp/-113 bp and -109 bp/-100 bp respectively.

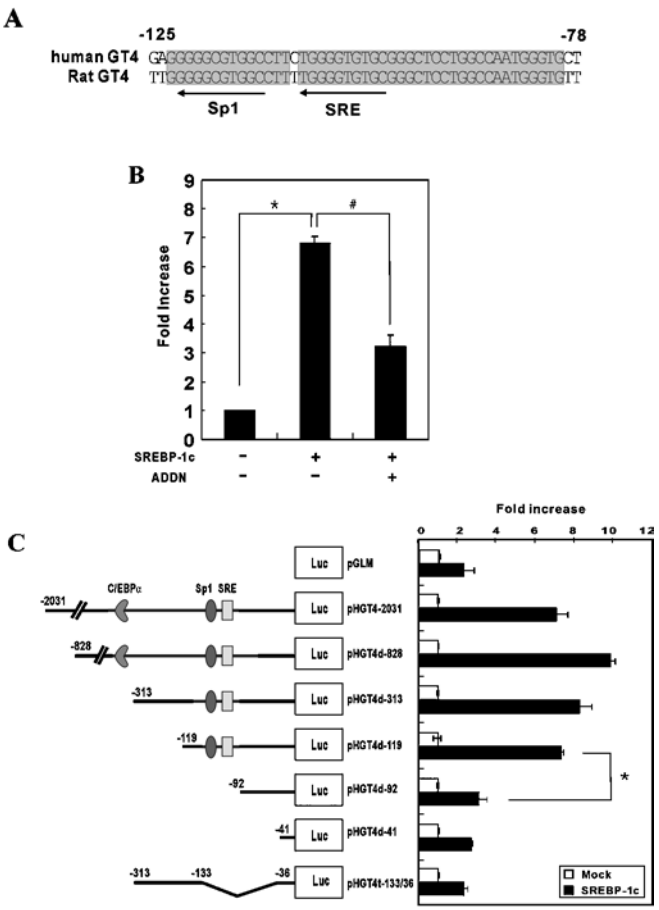

Figure 2 Localization of SRE in the human GLUT4 promoter

(**A**) Comparison of human and rat GLUT4 promoter sequences. (**B**) The effect of SREBP-1c on GLUT4 promoter activity. pHGT4-2031 promoter reporter (500 ng) was cotransfected with SREBP-1c (100 ng) or SREBP-1c plus ADDN into 3T3-L1 preadipocytes cell lines that were plated at a density of 2×10^5 cells/35 mm dish. Transient transfection, luciferase assays and measurement of total protein concentration of the lysates were performed as described in the Experimental section. $^{*}P < 0.001$, untreated versus SREBP-1; $^{\#}P < 0.05$, SREBP-1c versus SREBP-1c + ADDN. (**C**) The effect of deletion on the SREBP-1c-mediated GLUT4 promoter activity. Schematic diagram of serial deletion constructs of the GLUT4 promoter reporter (left) and SREBP-1c mediated GLUT4 promoter activity (right). The indicated numbers represent the number of nucleotides from the mRNA start codon. The promoter activities were measured by co-transfecting 100ng of SREBP-1c expression or empty vectors into 3T3-L1 preadipocytes. The results were normalized by the amount of total protein in the lysates, which was determined by the Bradford method and shown as the fold changes of luciferase activities compared to those of the control. Normalized luciferase activities are shown as the means ± S.D. of three independent experiments in triplicate. $^{*}P < 0.005$, pHGT4d-119 versus pHGT4d-92.

The functional role of Sp1 and SREBP-1 binding sites in the GLUT4 promoter

To analyse the functional role of the Sp1 and SREBP-1c binding sites on the activity of the GLUT4 promoter, we mutated the core binding sites for the respective transcription factors (Figure 5A). Figure 5(B) shows the effect of Sp1 and/or SREBP-1c overexpression on GLUT4 promoter activity. As shown, the ectopic expression of SREBP-1c strongly activated the GLUT4 promoter ($^{*}P < 0.001$, untransfected versus SREBP-1c). Sp1 also strongly activated the GLUT4 promoter ($^{**}P < 0.05$, untransfected versus Sp1). The co-transfection experiment of SREBP-1c with Sp1 suggested that Sp1 may play an additive role in the SREBP-1c mediated activation of the GLUT4 promoter ($^{\#}P < 0.001$ SREBP-1c versus SREBP-1c + Sp1). Co-transfection of SREBP-1c with the wild-type construct (pHGT4d-313) or mutants Ma, Mb or Mab to 3T3-L1 preadipocytes (Figure 5C) showed that the Sp1 mutant caused a decrease in SREBP-1c-driven GLUT4 promoter activation ($^{*}P < 0.05$, pHGT4d-313 versus Ma). The SRE mutant, Mb, also showed a decrease in the SREBP-1c-mediated GLUT4 promoter activation ($^{**}P < 0.05$, pHGT4d-313 versus Mb). Mutations at both sites (Mab) reduced the promoter activity to the basal level (Mab versus pGLM).

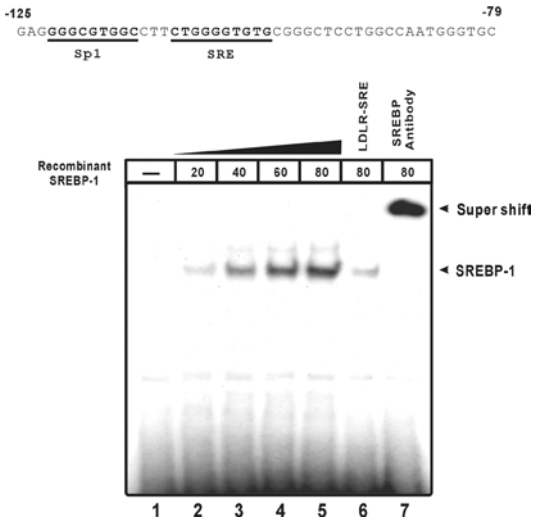

Figure 3 Identification of the SRE and binding of SREBP-1 in the human GLUT4 promoter

EMSA was performed with recombinant SREBP-1 protein in 4% (w/w) non-denaturing polyacrylamide gel. Then, 50000 cpm (0.1 pmole) of ^{32}P-labelled GLUT4 promoter fragments (−125/−79) containing putative SRE was incubated with 20–80 ng of recombinant SREBP-1 protein. The DNA–protein complexes are indicated by arrowheads. LDLR-SRE, SRE region on the promoter of the known low-density lipoprotein receptor.

SREBP-1c mediates GLUT4 gene expression in differentiated 3T3-L1 adipocytes

To confirm a direct role of SREBP-1c on GLUT4 expression, we treated differentiated 3T3-L1 adipocytes with 100 nM insulin and adenovirus containing GFP or ADDN. The endogenous GLUT4 gene expression is increased 4-fold by insulin and ADDN decreased GLUT4 mRNA levels in a dose-dependent manner, as shown by RT-PCR (Figure 6A) and real-time PCR (Figure 6B). The FAS promoter was used as a positive control for ADDN action (Figure 6C, $^{\#}P < 0.005$, GFP versus 200 MOI ADDN) [38]. As a negative control, mRNA levels of β-actin whose gene expression is known to be unaffected by ADDN [39] was measured by RT-PCR (Figure 6C). These data suggest that SREBP-1c acts as a *trans*-acting factor upregulating GLUT4 gene transcription in 3T3-L1 adipocytes.

SREBP-1c binding to the putative GLUT4-SRE is increased by refeeding and insulin treatment *in vivo*

Rats were fasted for 48 h and refed *ad libitum* to observe the effect of insulin on SREBP-1 binding to the putative SRE in the GLUT4 promoter. After 16 h, chromosomal DNA from adipose tissue was cross-linked by formaldehyde and an antibody specific to SREBP-1 was used to precipitate the chromatin fragment. We amplified a specific region of the GLUT4 promoter (−254 bp/−30 bp) by PCR (Figure 7A). The binding of SREBP-1 to the GLUT4 promoter was increased by refeeding (Figure 7B), suggesting that the increased concentration of plasma insulin elicited by refeeding

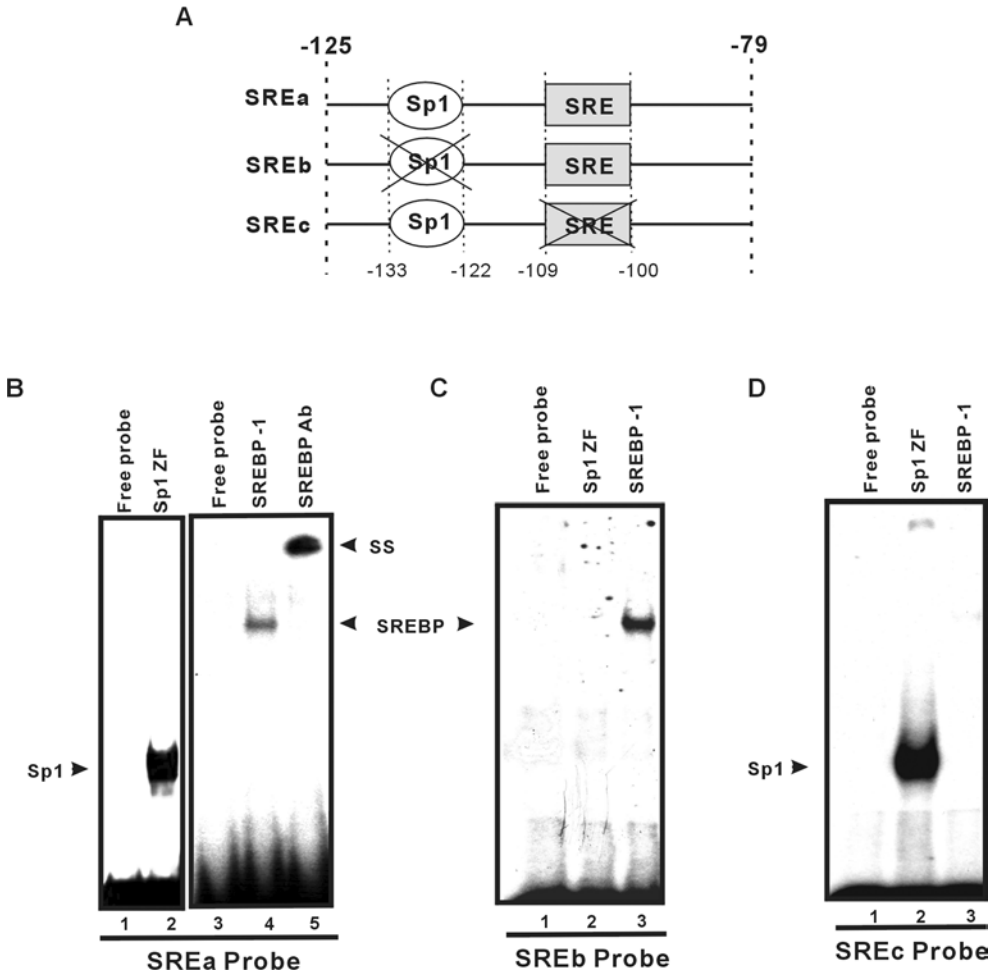

Figure 4 Binding of Sp1 and SREBP-1 to the GLUT4 promoter

(**A**) Schematic diagram of wild and mutated probes used in EMSA. The preparation of the site specific mutations are described in the Experimental section. (**B**–**D**) Direct and specific binding of the recombinant Sp1 zinc-finger (ZF) (80 ng) and SREBP-1 (200 ng) to each of the putative binding sites in the human GLUT4 promoter. The EMSA was performed using ^{32}P-labelled GLUT4 promoter oligonucleotides, Sp1 and SREBP-1. SS, supershift.

might have increased the binding of SREBP-1 to the GLUT4-SRE in the epididymal WAT. To confirm this effect of insulin on the SREBP-1c binding to the GLUT4-SRE, we administered insulin to STZ-induced diabetic rats and performed *in vivo* ChIP assays (Figure 7C). As shown, the binding of SREBP-1c to the SRE is restored by insulin treatment, suggesting that the insulin–SREBP-1c pathway plays a role in the activation of GLUT4 gene expression.

DISCUSSION

In T2DM or obesity, the amount of total GLUT4, as well as insulin-mediated recruitment of GLUT4 from intracellular vesicles to the plasma membrane, is reduced [40,41]. Although adipose tissue accounts for only a small fraction of glucose disposal [42], adipose specific knockout mice of the GLUT4 gene display insulin resistance and glucose intolerance [43], suggesting that the adipose tissue GLUT4 might play a critical role in the glucose homoeostasis *in vivo*. This observation is further supported by the finding that overexpression of GLUT4 in the adipose tissue improves glucose disposal and insulin sensitivity *in vivo* [44]. The expression of GLUT4 is reduced in rodent models of diabetes [45,46] and in the adipose tissues of obese and T2DM subjects [16,17], indicating that GLUT4 in the adipose tissue is linked to insulin resistance. Accordingly, understanding mechanisms involved in the regulation of GLUT4 gene expression might be critical in enhancing insulin sensitivity and reducing the hyperglycaemia in T2DM. Despite the significant role of GLUT4 in glucose metabolism, the molecular mechanisms underlying the transcriptional regulation of GLUT4 are poorly understood.

SREBP-1c is involved in the effect of insulin on the transcription of genes involved in lipid and carbohydrate metabolism [38,47,48]. Notably, SREBP-1c expression is decreased in the liver during fasting and is increased by refeeding [49,50]. Thus, it is tempting to speculate that the insulin–SREBP-1c pathway in the adipocytes is involved in the up-regulation of GLUT4 gene expression. However, recent work using FIRKO (fat-specific insulin receptor knock-out) mice showed that these mice display a marked reduction in adipogenic transcription factors like CCAAT/enhancer binding protein-α and SREBP-1c [51]. In this animal model, reduction in GLUT1 but not in GLUT4 protein levels was observed suggesting that GLUT1, rather than GLUT4, expression is insulin-responsive. These results argue against the current concept that GLUT4 is a direct target gene of insulin and instead suggest that insulin regulates GLUT4 gene expression through an indirect mechanism. Therefore it is likely that GLUT4

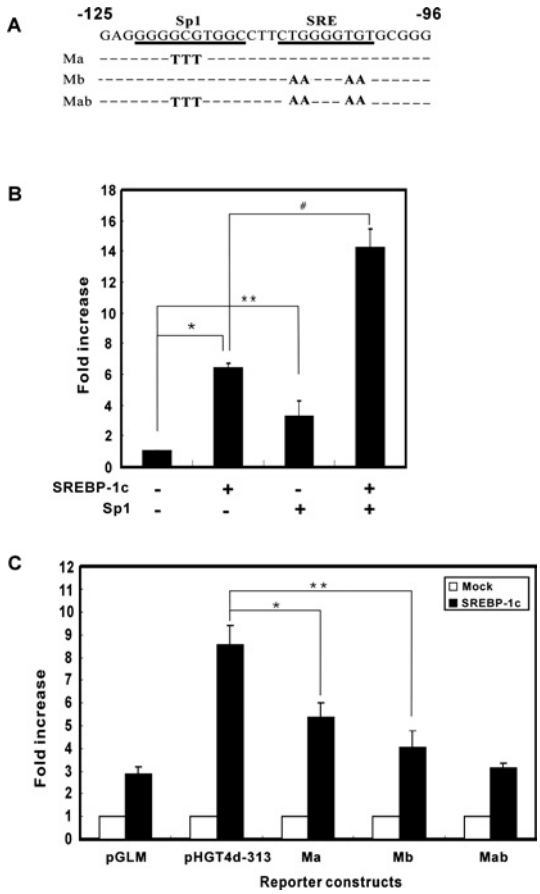

Figure 5 The functional relationship between Sp1 and SREBP-1 in the GLUT4 promoter

(**A**) The sequences of wild-type and the mutated construct of putative Sp1 and SREBP-1c binding sites. The mutated bases are represented by bold characters. (**B**) The effect of the SREBP-1c and Sp1 on human GLUT4 promoter activity. SREBP-1c or Sp1 expression vector was transfected to 3T3-L1 preadipocytes. The luciferase activities were represented as fold changes compared with the control group. $^{*}P < 0.001$, untransfected versus SREBP-1c; $^{**}P < 0.05$, untransfected versus Sp1; $^{\#}P < 0.001$, SREBP-1c versus SREBP-1c + Sp1. (**C**) GLUT4–luciferase constructs containing mutations in the Sp1 or SREBP-1c were generated as described in the Experimental section. 3T3-L1 preadipocytes were co-transfected with each promoter fragment linked to luciferase with or without SREBP-1c expression vectors. Ma, promoter reporter of mutated Sp1 sequence. Mb, promoter reporter of mutated SRE sequence. Mab, promoter reporter of double mutated Sp1 and SRE sequences. Values are the means ± S.D. of three independent experiments performed in triplicate. $^{*}P < 0.05$, pHGT4d-313 versus Ma, $^{**}P < 0.05$, pHGT4d-313 versus Mb.

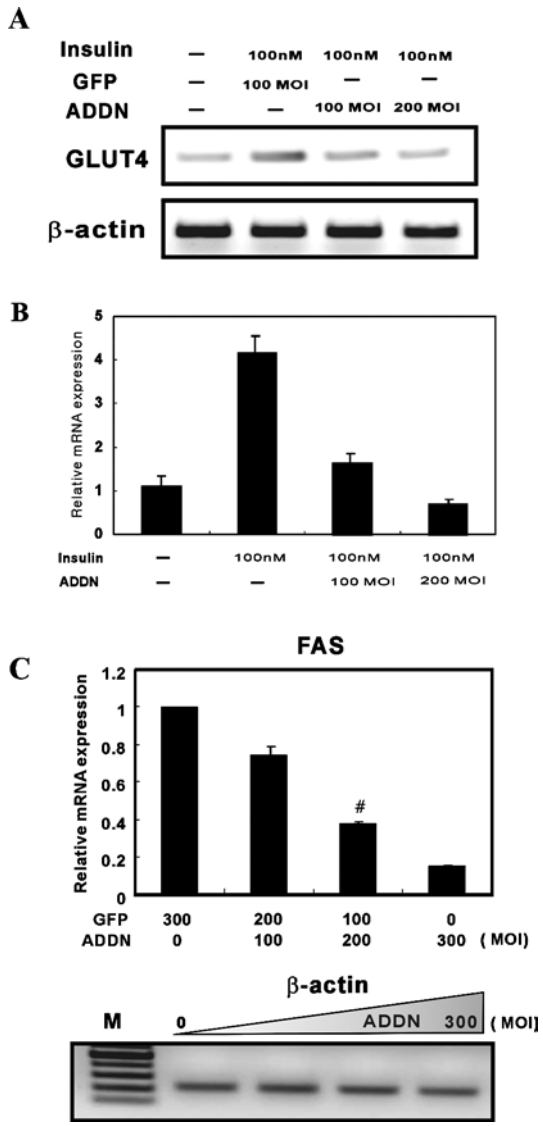

Figure 6 SREBP-1c mediates GLUT4 gene expression in differentiated 3T3-L1 adipocytes

3T3-L1 adipocytes were treated with insulin (100 nM) and ADDN or null virus (adeno-GFP) at the indicated MOI for 16 h at 37 °C with serum-free media and replaced with media including serum. Total RNA was extracted for the measurement of GLUT4 mRNA using (**A**) RT-PCR or (**B**) real-time PCR. (**C**) The effect of ADDN on FAS gene expression. The mRNA level was normalized to that of α-tubulin in real-time PCR. β-Actin gene expression measured by RT-PCR was used as a negative control. M, DNA marker. Values are the means ± S.D. of three independent experiments performed in triplicate. $^{\#}P < 0.005$, GFP versus ADDN (200 MOI).

is regulated by the hormonal/metabolic milieu that develops as a result of insulin deficiency.

In the present study, we observed that a fasting/refeeding protocol caused an increase in SREBP-1c levels (Figure 1A) and insulin administration to STZ-diabetic rats induced GLUT4 mRNA expression in epididymal WAT (Figure 1B). These results are supported by Olson et al. [52] who showed the induction by insulin in GLUT4-overexpressing transgenic mice of GLUT4 mRNA in WAT and brown adipose tissue (BAT). Refeeding increased mRNA levels of SCD1 and FAS which are known target genes of SREBP-1c (Figure 1C). However, there is a difference in the induction time point between SCD1 (6 h) and FAS mRNA (12 h). This result does not match with the onset of SREBP-1 mRNA induction. Insulin activates SREBP-1 gene transcription as well as the processing of the premature form to the mature form [53], therefore it could be possible that a small portion of the pre-existing premature SREBP-1 can be converted to the mature form, which in turn activates the target genes before a substantial amount of SREBP-1 is transcribed. Indeed, in the condition where the premature form of SREBP-1c is increased, as in adipocyte differentiation [54], insulin is shown to promote the processing of SREBP-1c to the mature form resulting in the activation of SREBP-1c target genes. During the refeeding period, the mature form of SREBP-1 present in the nuclear fraction of WAT, was increased after 6 h (Figure 1D), suggesting that insulin may be involved in the processing of SREBP-1. From this data, it is assumed that the appearance of mature SREBP-1 protein coincides with or precedes GLUT4 mRNA induction.

Endogenous GLUT4 gene expression was increased by exogenous SREBP-1c in the differentiated 3T3-L1 adipocytes (Figure 1E). These results indicate that SREBP-1c acts as a positive

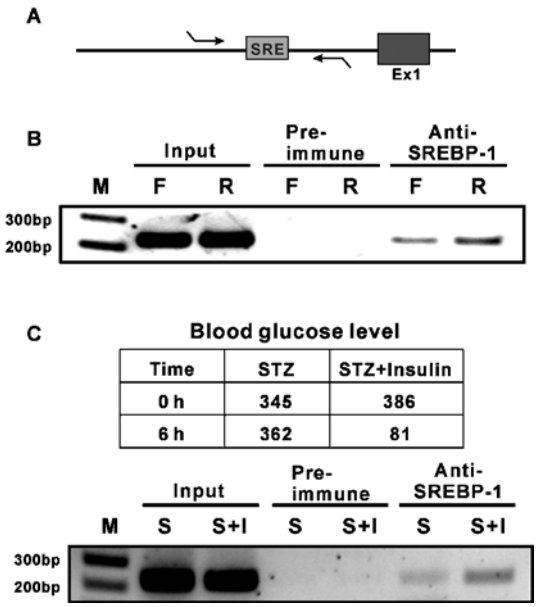

Figure 7 SREBP-1c binding to putative GLUT4-SRE is increased by refeeding and insulin treatment *in vivo*

(**A**) Scheme of amplifying the GLUT4 promoter in the ChIP assay. The region between −280 to −55 was amplified. (**B**) For the ChIP assay, chromatin extracted from epididymal WAT was precipitated by SREBP-1 antibody and GLUT4-SRE was amplified by PCR. The amount of chromosomal DNA used for the precipitation reaction was normalized by input chromatin (1/100th of chromosomal DNA used for precipitation). F, fasted; R, refed. (**C**) The effect of insulin on the binding of SREBP-1 to the GLUT4 promoter in adipose tissue of STZ-induced rats. Insulin depleted diabetic rats were prepared by STZ. For the insulin treatment group, 0.1 mM/kg of insulin was administrated to STZ-induced diabetic rats. The ChIP assay is representative of three independent experiments. Blood glucose levels for the animal groups are shown in the Table. F: 48 h fasted, R: refeeding rats, S: STZ, I: insulin.

trans-acting factor for the GLUT4 gene. Thus, it is speculated that insulin upregulates GLUT4 gene transcription and SREBP-1c functions as a mediator of insulin action in the WAT of rats. In the differentiated 3T3-L1 adipocytes, insulin induced GLUT4 mRNA levels and transduction of ADDN to the insulin-treated 3T3-L1 adipocytes resulted in the decrease in GLUT4 mRNA (Figures 6A and 6B), indicating that insulin-induced SREBP-1c could activate GLUT4 gene expression.

An *in vivo* ChIP assay showed that the refeeding or insulin treatment of STZ-diabetic rats increased the binding of SREBP-1c to the putative SRE in the GLUT4 promoter (Figure 7). In addition, we showed that the upregulation of GLUT4 gene expression in these states is directly mediated by the binding of SREBP-1c to a conserved sequence (−109/−100, TGGGGTGTG) in the GLUT4 promoter. This study provides evidence that GLUT4 gene up-regulation could occur directly by the action of SREBP-1c and expands the current understanding of the transcriptional control of GLUT4 gene expression.

How does one explain the observation that GLUT4 is up-regulated by SREBP-1c, a gene whose expression is not insulin-responsive? In fact, the transcriptional control of the SREBP-1c gene has been shown to be influenced by the combinatorial effects of insulin and nutritional alterations [55,56]. Thus, it is possible that the upregulation of GLUT4 expression could be induced not only by insulin-induced SREBP-1c but also by several transcription factors that might up-regulate the SREBP-1c gene. Indeed, the SREBP-1c gene is activated by the liver X receptor in adipocytes [57,58]. These results suggest that the GLUT4 gene can be activated by SREBP-1c which itself can be up-regulated either by the independent or indirect action of insulin. Indeed, Matsuzaka et al. [59] showed that SREBP-1c regulation is independent of insulin in a situation where carbohydrate supply is sufficient in the livers of STZ-treated mice. In addition, we and others have reported that SREBP-1c gene expression was increased by glucose [36,60]. Based on these observations, we suggest that SREBP-1c can activate GLUT4 gene expression by directly binding to the SRE in the GLUT4 promoter.

The current study provides evidence that up-regulation of the GLUT4 gene can be mediated by the direct action of SREBP-1c and adds to the current understanding of the transcriptional control of GLUT4 expression. Further studies are needed to identify additional molecules that govern the SREBP-1c-mediated activation of the GLUT4 gene.

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