Mechanism of Transcriptional Regulation of Glucose Transporter Type Ⅳ Isoform in Adipose Tissue

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Mechanism of Transcriptional Regulation of Glucose Transporter Type Ⅳ Isoform in Adipose Tissue

Directed by Professor Yong-Ho Ahn

The Master's Thesis submitted to the Department of Medical Science , the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

Seung-Youn Kang

June 2004

This certifies that the Master's Thesis of

Seung-Youn Kang is approved.

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Kyung-Sup Kim

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Ho-Sung Kim

The Graduate School

Yonsei University

June 2004

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Seung-Youn Kang

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ABSTRACT

Mechanism of transcriptional regulation of glucose transporter type Ⅳ isoform in adipose tissue

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GLUT4 is an insulin-responsive glucose transporter which is expressed predominantly in muscle and adipose tissues. Although GLUT4 has been shown to play a critical role in maintaining systemic glucose homeostasis, the mechanisms of transcriptional regulation are not incompletely understood, in detail.

In this study, we showed that GLUT4 and SREBP-1c expression in adipose tissue is stimulated by food intake at transcriptional level. The transient transfection with GLUT4 - luciferase serial deletion constructs in 3T3-L1 preadipocytes revealed that the region from -119 to -41 contains functional elements required for SREBP-1c binding

which mediates GLUT4 transcriptions by insulin and diet. Electrophoretic mobility shift assays (EMSA) demonstrated that SREBP-1, Sp1, and NF-Y bound to the human GLUT4 promoter. Introduction of mutations of putative Sp1, SRE1, and NF-Y sites decreased the GLUT4 promoter activity by SREBP-1c. Taken together, GLUT4 is under the transcriptional control of insulin mediated SREBP-1c signaling. Moreover, Sp1 and NF-Y play a critical role in the activation of human GLUT4 gene expression by SREBP-1c.

Key words : GLUT4, adipose tissue, Sterol regulatory element-binding protein-1c (SREBP-1c), transcriptional regulation , Sp1, NF-Y

Mechanism of transcriptional regulation of glucose transporter type Ⅳ isoform in adipose tissue

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I. INTRODUCTION

Glucose functions as a precursor for the synthesis of glycoproteins, triglycerides, and glycogen and provides an important energy source. Because glucose is a polar molecule, it does not readily diffuse across the hydrophobic plasma membrane, and therefore, specific carrier molecules exist to mediate the specific uptake of glucose.[1,2]

Glucose uptake in eukaryotic cells is mediated by the GLUT family of glucose transport proteins. Thirteen isoforms of GLUT proteins have been identified and cloned, and their tissue specificity has been extensively studied. The GLUT4 isoform is expressed in insulin target tissues, i.e. adipose tissue and muscle, where it mediates an increase in

glucose uptake in response to acute insulin stimulation.[3, 4, 5] Although GLUT4 has been shown to play a critical role in maintaining systemic glucose homeostasis, the mechanisms of transcriptional regulation are not well understood.

SREBP-1c (sterol regulatory element binding protein-1c, also known ADD1, adipocyte determination and differentiation dependent factor 1) is a member of SREBPs that regulate the transcription of many genes involved cholesterol and fatty acid synthesis.[6, 7] SREBPs are members of the basic helix-loop-helix leucine zipper family of transcription factors that regulate fatty acid and cholesterol synthesis.[8, 9, 10] SREBP proteins are initially bound to the rough endoplasmic reticulum membrane and form a complex with SREBP-cleavage activating protein, a sterol-sensing molecule.[11, 12] Upon sterol deprivation, SREBP is cleaved to liberate the amino terminal portion containing a basic helix-loop-helix leucine zipper domain (nuclear SREBP) and enters nucleus, where it binds to specific sterol response elements or palindromic sequences called E-boxes in the promoters of target genes.[13, 14, 15] Three isoforms of SREBP, -1a, -1c, -2 are known. SREBP-1a and -1c are derived from a single gene through the use of alternate promoters and SREBP-2 from a different gene. SREBP-1a is the more common isoform and is a stronger activator of transcription with a wider range of target genes than SREBP-1c because of a longer transactivation

domain.[16] Transgenic mouse studies have shown that SREBP-1c plays a more active role in regulating the transcription of genes involved in fatty acid synthesis than those involved in cholesterol synthesis, whereas SREBP-1a activates both.[16, 17] SREBP-2 is known to be actively involved in the transcription of cholesterogenic enzymes.[18] It has been shown that all cultured cells analyzed to date exclusively express SREBP-2 and the SREBP-1a isoform, whereas most organs, including the liver, express predominantly SREBP-2 and the -1c isoform.[19]

Recent study demonstrates that SREBP-1c mRNA is more abundant in adipose tissue than in skeletal muscle in humans.[20] Also, the induction of SREBP-1c mRNA by glucose leads to increased mature protein in the nucleus, thus providing a potential mechanism for the up-regulation of lipogenic genes by glucose *in vivo*.[21] SREBP-1c is known to be an important mediator of transcriptional effects of insulin in adipocytes, and C/EBPβ is under the direct control of SREBP-1c.[22]

In this study, attempts were made to investigate a role of SREBP-1c on the transcriptional activation of GLUT4. Through this study, the binding site of SREBP-1c in the human GLUT4 promoter is identified and roles of accessory transcriptional factors necessary for SREBP-1c actions were identified.

II. MATERIALS AND METHODS

1. Materials

All reagents for cell cultures, Lipofectamin PLUS, and TRIzol were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA). Rapid-Hybrid buffer was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Bradford assay kit was purchased from Bio-Rad (Hercules, CA, USA). [α-^{32}P] dCTP (3.000 Ci/mmole) and [γ-^{32}P] ATP (6,000 Ci/mmole) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). NF-YA antibody was purchase from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). SREBP-1 antibody was a gift from Dr. Kyung-Sup Kim (Yonsei University College of Medicine). Dexamethasone and isobutyl-methylxanthin were from Sigma (Sigma-Aldrich Co.).

2. Animal and diet

Male Sprague-Dawley rats weighing 150g were used for all experiments. For fasting and refeeding study, rats were fasted for 24 h and refed with fat-free high-carbohydrate diet for 6, 12, and 24 h. The fat-free high-carbohydrate diet contained 82% (w/w) carbohydrates (74% starch, 8% sucrose), 18% (w/w) casein, 1% (w/w) vitamin mixture,

and 4% (w/w) mineral mixture. All the materials for diet were purchased from Harlan Teklad Co. (Madison, WI, USA).

3. Cell culture and differentiation

3T3-L1 preadipocytes (ATCC number CL-173) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 unit/ml penicillin streptomycin. The cells were induced to differentiate into the adipocyte phenotype by treating confluent cells with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich Co., USA), 1 μM dexamethasone (Sigma-Aldrich Co. USA) and 10 μg/ml insulin (Sigma-Aldrich Co. USA) for 2 days, followed by treatment with 10 μg/ml insulin in DMEM for 2 days. The cells were then maintained in DMEM with 10% FBS every other day.

4. Northern blot hybridization of mRNA

Total RNA was isolated from fat of rat, which was fasted or refed for indicated periods, by TRIzol (Invitrogen, USA) according to the manufacturer's protocol. 20 μg for each sample were denatured with RNA sample loading buffer (20 mM MOPS, pH 7.0, 2 mM sodium acetate, 1mM EDTA, 8% (v/v) formaldehyde, 50% (v/v) formamide), and subjected to electrophoresis in a 0.9% denaturing formaldehyde agarose gel, and transferred to Nylon membrane (Schleicher & Schuell,

Germany). The cDNA fragment of human GLUT4 gene was labeled with [α-^{32}P]dCTP using Rediprime Labeling Kit (Amersham Pharmacia Biotech, Bucks, U.K.) and used as a probe. The membranes were hybridized with the probe for 4 h at 65℃ with Rapid hybrid buffer (Amersham Pharmacia Biotech). After hybridization, the membrane washed twice high salt washing buffer (0.1% SDS, 2X SSC) at room temperature for 15min in twice followed by low salt washing buffer (0.1% SDS, 0.2X SSC) at 65℃ for 15min in twice. The membrane was exposed to X-lay film with intensifying screen at -70℃.

5. Construction of plasmids

The human pG4CHⅢ_GLUT4 promoter was a gift from Dr. Ann Louise Olson (The University of Oklahoma). To generate the human GLUT4 luciferase reporter plasmid, the *Sac* I -*Xba* I fragment of the human pG4CHⅢ_GLUT4 was inserted into pGLM which was eliminated E-box from pGL3 basic vector (Promega, Madison, WI, USA), and named pHGT4-2031/+61. 5' serial deletion of human GLUT4 promoter reporter constructs pHGT4d-828, pHGT4d-313, pHGT4d-119, pHGT4d-92, and pHGT4d-41 were constructed by amplifying human GLUT4 promoter regions of -828/+61, -313/+61, -119/+61, -92/+61, and -41/+61, respectively, and subcloning into pGLM vector. The Sp1, SRE1, and NF-Y truncated mutant, pHGT4t-215/+61, which -133/-36

region was truncated from pHGT4d-313 was amplified by PCR using the following primer (sense : 5'-CCAAACTCTAAACGGCTTCTCGCG TCTTTTCCC-3', antisense : 5'-GACGCGAGAAGCCGTTTAGAGTTTG GCTGGAGT-3'), and subcloned the *Kpn* I -*Xho* I of PCR fragment into the same restriction sites of a pGLM vector. Mutant constructs pHGT4-Sp1m (Ma), pHGT4-SREm (Mb), pHGT4-NF-Ym (Mc), pHGT4-Sp1m/SREm (Mab), pHGT4-SREm/NF-Ym (Mbc), and pHGT4-Sp1m/NF-Ym (Mac) were produced by substitution mutations into pHGT4d-313 using site-directed mutagenesis. The sequences of all constructs were confirmed by DNA sequencing.

6. Transient transfection

3T3-L1 cells were plated in six-well tissue culture plates at a density of 1 X 10^6 cells/well in 2 ml of medium. After a 20 h attachment period, cells were transfected with the indicated plasmids by using Lipofectamine PLUS according to the manufacturer's protocols. Briefly, the plasmid DNA and 4 μl of PLUS reagent were mixed in 100 μl of OPTI-MEM and then added to 100 μl of OPTI-MEM containing 2 μl of Lipofectamine reagent. The total amount of DNA in each transfection was adjusted to the same amount by addition of mock vector plasmid. The cells were washed with PBS and supplied with 800 μl of OPTI-MEM. After 15 min of incubation, Lipofectamine-DNA mixtures were

added into wells. The cells were transfected for 3 h with the plasmid, then grown in DMEM supplemented with 10% FBS and 100 μg/ml penicillin streptomycin. After 48 h, the cells were harvested and lysed by 150 μl of reporter lysis buffer (Promega, Madison, WI, USA), and cell debris was removed by centrifugation and the supernatant was collected. Luciferase assays were conducted with 20 μl of cell extracts and 50 μl of luciferase assay reagent (Promega, Madison, WI, USA). Total proteins of lysates were determined by Bradford method. Luciferase activities were normalized by amount of total proteins because the expression of β-galactosidase is suppressed by overexpression of SREBP-1. Each transfection was performed in triplicate and repeated three to five times.

7. Nuclear extracts preparation

Rats fasted or refed were sacrificed and epididymal adipose tissue was weighed. About two grams of adipose tissue were homogenized with 0.3 M sucrose homogenation buffer (15 mM HEPES, pH 7.9, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.3 M sucrose, 0.5 mM DTT, 0.5 mM PMSF, 5 g/ml protease inhibitor cocktail containing pepstatin A, leupeptin, chymostatin, and antipain) by polytron homogenizer. The cell lysate was filtered through gauze and added upon the 0.9 M sucrose buffer for nuclear isolation. The nucleus was pelleted by centrifugation at 3,000 rpm for 5 min at 4C. The nucleus was resuspended in nuclear storage buffer (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 5 g/ml protease inhibitor cocktail) and after brief centrifugation, the pellet was resuspended in 1X NUN buffer (1 M urea, 0.3 M NaCl, 1% NP40, 25 mM HEPES, pH 7.6, 1 mM DTT, 5 g/ml protease inhibitor cocktail) and shaken for 30 min at 4℃ for extraction of nuclear protein. After centrifugation at 12,000 rpm for 15 min at 4℃, the samples were collected and stored at - 70℃. The protein concentration was determined by Bradford method.

8. Electrophoretic mobility shift assay (EMSA)

Ten pmoles of a single-stranded oligonucleotide was labeled at 5' end by incubation with T4 polynucleotide kinase (TaKaRa, Shiga, Japan) and [ɣ-^{32}P]ATP at 37℃ for 60 min. Five molar excess of complementary oligonucleotide was added to reaction mix, and heated to 95℃ for 3 min, followed by cooling down to room temperature. After annealing reaction, free isotope was removed by passing the reaction mix through Sephadex G50 spin-column. For EMSA, the probes (5 X 10^5 cpm) purified recombinant SREBP-1 (120 ng), recombinant GST-Sp1-zinc finger domain (120 ng) or fat nuclear extract (1 μg) in final volume of 20 μl containing 10 mM HEPES, pH 7.9, 60 mM KCL, 1 mM DTT, and 1 μg of poly(dI-dC). The binding reaction was carried for 30 min on ice. Protein-DNA complexes were resolved from the free probe by electrophoresis at 4℃ on a 4% (w/v) polyacrylamide gel in 0.25 X TBE buffer (1 X TBE contained 9 mM Tris, 90 mM boric acid, and 20 mM EDTA, pH 8.0). After electrophoresis, polyacylamide gel was dried and exposed to X-ray film at -70℃ with an intensifying screen. The oligonucleotides used in EMSA were shown in Table 1.

Table 1. The sequences of the oligonucleotides used in this study

Oligonucleotide	Sequence (5'→3')
SREa	GCCTTCTGGGGTGTGCGGGCTCCTGGCCAATGGG TGCTGTGAAGG
SREb	GAGGGGGCGTGGCCTTCTGGGGTGTGCGGG
SREc	TCCTGGCCAATGGGTGCTGTGAAGGGCGTG
SREd	GAGGGGGCGTGGCCTTCTGGGGTGTGCGGGCTCC TGGCCAATGGG
SREe	GAGGGGGCGTGGCCTTCTGGGGTGTGCGGGCTCC TGGCCAATGGGTGCTGTGAAGGG
Ma	CAGGCGGGGG**TTT**TGGCCTTCTGGGGTGTGC
Mb	TGGCCTTC**AA**GGG**AA**TGCGGGCTCCTGGCCAATGG
Mc	GGCTCCTGGC**GT**ATGGGTGCTGTGAAGGGCGT

Mutated regions are marked by bold letters.

9. Statistical analysis

All transfection studies were performed in 3-5 separate experiments, where triplicate dishes were transfected. Data are expressed as means ± S.D. and compared by the Student's t-test.

III. RESULTS

1. Dietary regulation of GLUT4 and SREBP-1c in adipose tissue

Expression of GLUT4 is altered in response to changing metabolic conditions. A high-fat diet leads to reduction in GLUT4 levels in both skeletal muscle and adipose tissue.[23] In rats, fasting results in reduced GLUT4 expression in adipose tissue, but increased expression in skeletal muscle.[24] Moreover, SREBP-1 is known to be regulated by glucose at the transcriptional level.[21] To examine the expression of GLUT4 and SREBP-1c in mRNA level in fasted and refed rats, northern blot hybridization was carried out. A low expression level of the GLUT4 transcript was detected in 1 day fasted and 6 h refed status, but a robust induction of the GLUT4 transcript was observed later around 12 h and 24 h refeeding. The expression of SREBP-1c was also slightly induced in 1 day fasted and 6 h refed status, and was markedly increased at 12 h and 24 h after refeeding (Fig. 1). This result suggests that the expression level of GLUT4 and SREBP-1c transcript of adipose tissues is controlled during fasting and refeeding cycle.

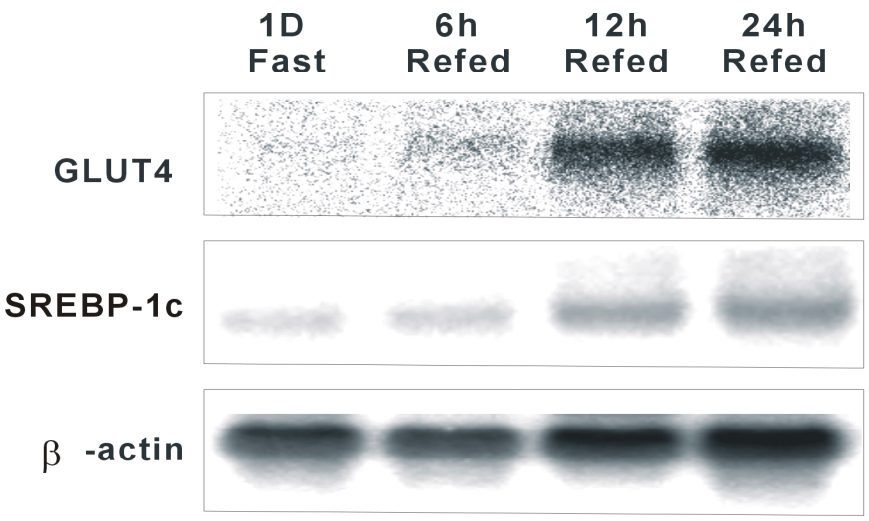


Figure 1. Time-course changes of GLUT4 and SREBP-1c mRNA in the adipose tissue during fasting and refeeding status. Northern blot analysis of GLUT4 and SREBP-1c mRNAs in the rat adipose tissues. Total RNA isolated from adipose tissues of each group was subjected to formaldehyde agarose gel electrophoresis. RNA in the gel was transferred to nylon membrane and hybridized to ^{32}P-labeled cDNAs for GLUT4, SREBP-1c, or β-actin.

2. SREBP-1c activates human GLUT4 promoter

In order to know the responsiveness of human GLUT4 promoter to SREBP-1c, luciferase reporter construct under control of human GLUT4 promoter, pHGT4-2031/+61, was prepared and transfected into 3T3-L1 preadipocytes with or without overexpression of SREBP-1c. As shown in Fig. 2A, luciferase activity of pHGT4 was increased 5-fold by addition of SREBP-1c. However, dominant negative form of SREBP-1c suppressed the luciferase activity of pHGT4-2031/+61 construct by 40% (Fig. 2B). This result suggests the presence of SRE in the human GLUT4 promoter construct and the preference of the SRE to SREBP-1c.

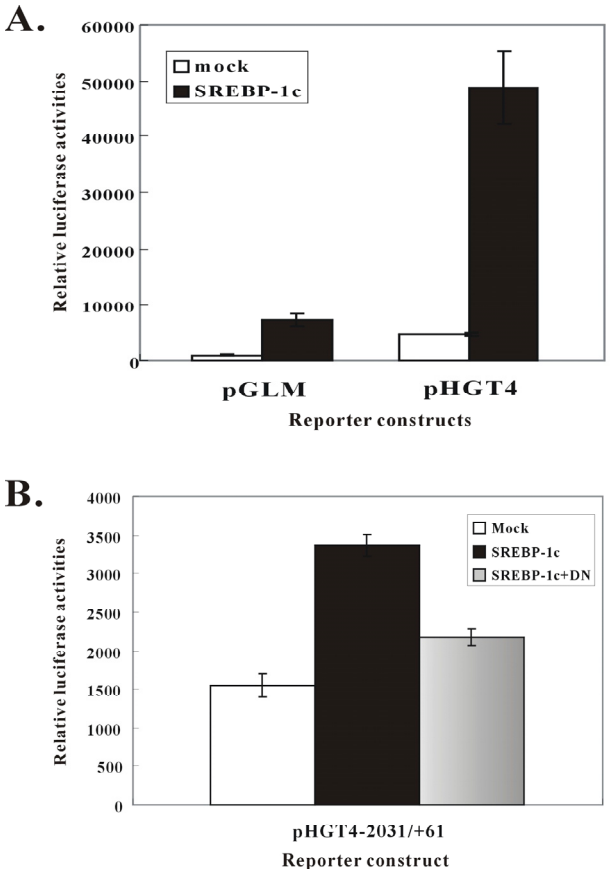


Figure 2. SREBP-1c activates human GLUT4 promoter (A) pHGT4-2031/+61 and control pGLM vector were transfected into 3T3-Li preadipocytes. Expression plasmid of SREBP-1c (black bar) or control plasmid SV_SPORT (white bar) was cotransfected with the reporter. **(B)** Expression plasmids of SREBP-1c and SREBP-1c dominant negative form (gray bar) were cotransfected with pHGT4-2031/+61. The data represent the mean ± S.D. of three independent experiments performed in triplicate.

3. Human GLUT4 promoter sequence

To study the effect of additional transcription factor involved in GLUT4 gene expression, we have sequenced and analyzed 2 Kb upstream from the transcription initiation site of the human GLUT4 gene (data not shown). Moreover, we compared sequences between human and rat GLUT4 gene promoter regions (Fig. 3A).

Computer assisted search (the TFSEARCH program based upon the TRANSFAC data base) of the 5'-flanking region of the human GLUT4 promoter revealed potential *cis*-elements that may play roles in the human GLUT4 transcriptional regulation. Inverted Sp-1 binding site was found at -122/-113 bp region. Adjacent to the Sp1 site, there was a sequence similar (7 of 10 bp) to one of authentic sterol regulatory elements, inverted SRE1 (CTGGGGTGTG), which was found in the LDL receptor gene promoter.[25, 26] Moreover, NF-Y-binding site(CCAAT) was found at -88/-84 bp region. Following the NF-Y-binding site was the inverted SRE2 from between -81 and -73 bp region (Fig. 3B).

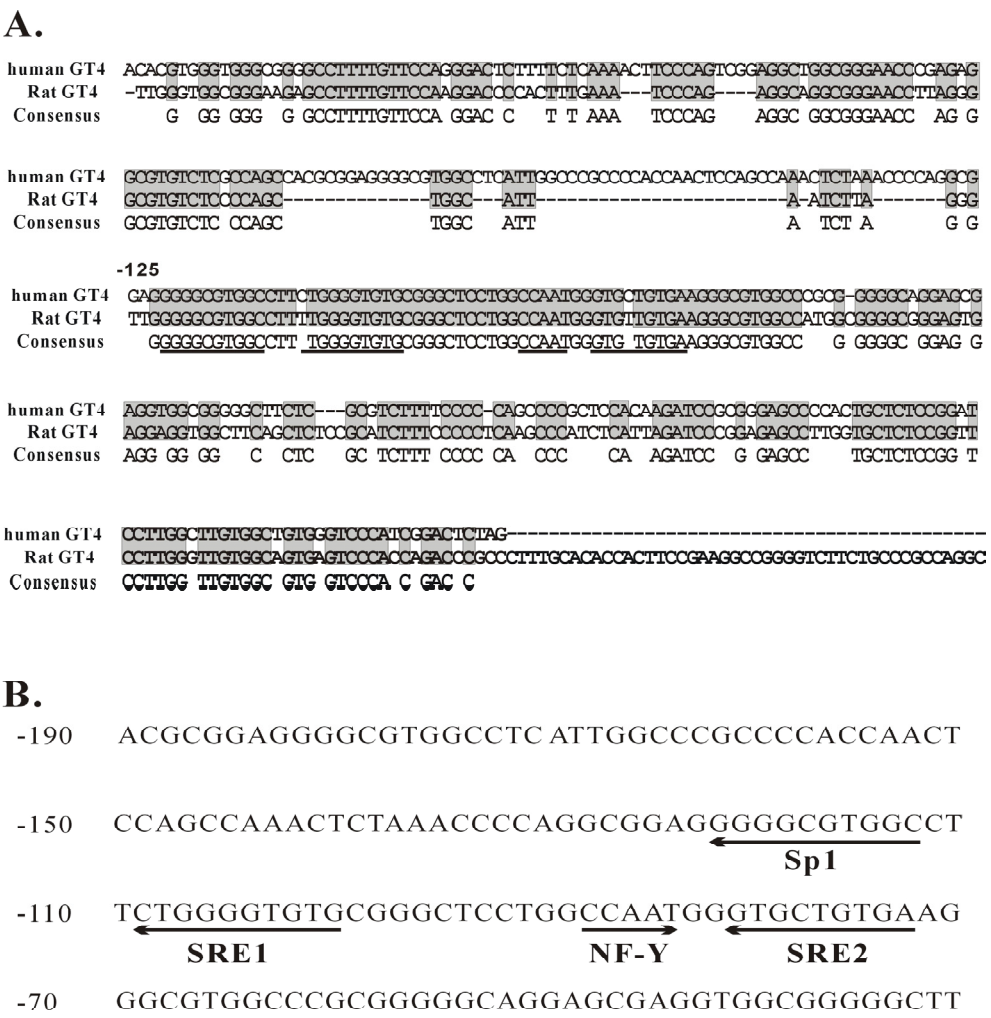


Figure 3. Homology comparison and consensus sequence of human GLUT4 gene (A) Alignment of human and rat GLUT4 promoter (B) The putative consensus sequences of SREBP-1c, Sp1, and NF-Y binding sites. The putative regulatory *cis*-elements are indicated by the *arrows*.

4. Localization of SREBP-1c responsive region in human GLUT4 promoter

To identify the functional SRE involved in regulating the human GLUT4 promoter activity, the transient transfection assays were performed using serial deletion constructs (from -2031, -828, -313, -119, -92, -41 to +61, shown in Fig. 4A). As shown in Fig. 4B, pHGT4-2031, pHGT4d-828, pHGT4d-313, and pHGT4d-119 were activated by cotransfection of pSV_SREBP-1c. However, deletion of the sequences down to -92 (including putative Sp1-binding site and SRE1) suppressed the SREBP-1c driven promoter activity. The deletion of the region from -92 to -41 almost completely abolished the basal promoter activity. Further decrease of promoter activity in pHGT4d-41 was thought to be resulted from removing the binding site of basal transcription machinery as well as NF-Y and SREBP-1c. When the region from -134 to -35 (not including putative Sp1-binding site, SRE1, NF-Y binding site, and SRE2) was truncated from pHGT4d-313, the promoter activity was decreased by 59% (Fig. 4C). This data suggests that the region from -119 to -92, which may contain the putative SRE, plays an important role in mediating responsiveness of human GLUT4 promoter to SREBP-1c.

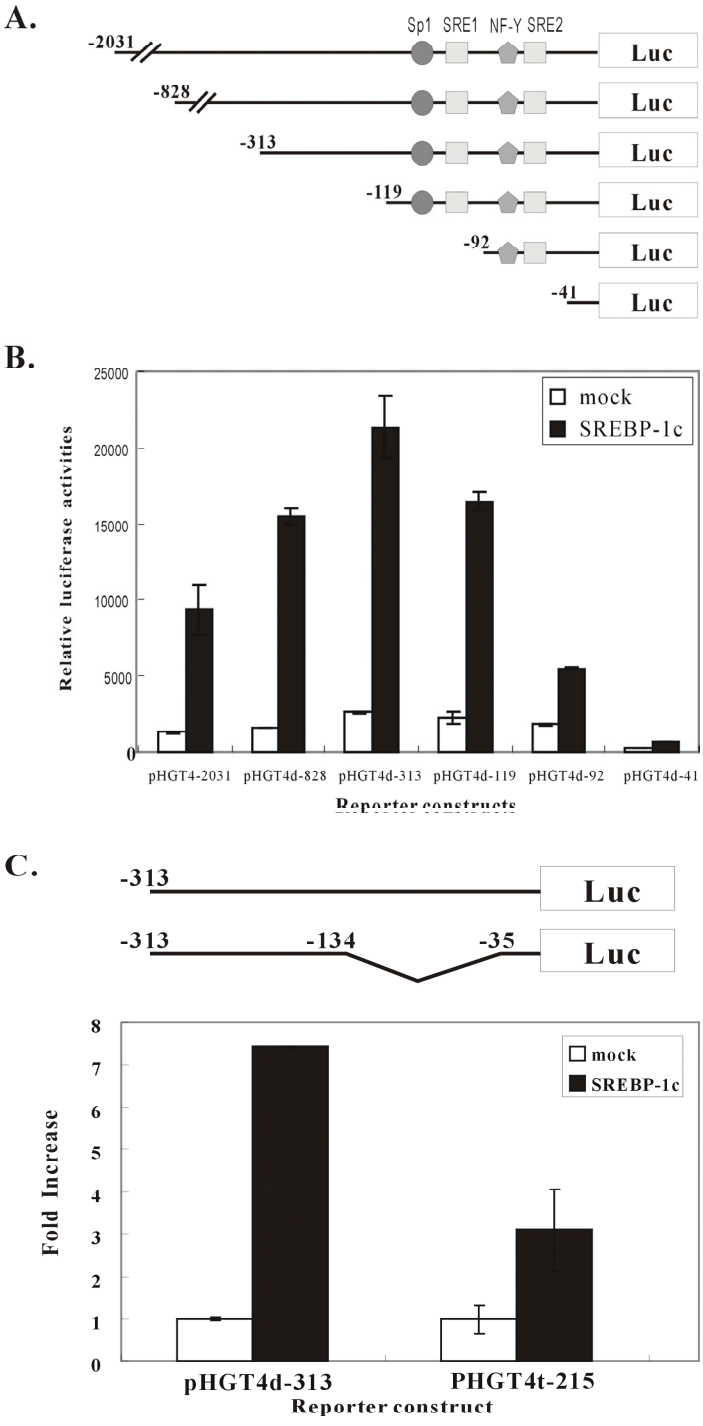

A.
Sp1 SRE1 NF-Y SRE2
-2031
-828
-313
-119
-92
-41
Luc
B.
mock
SREBP-1c
Relative luciferase activities
25000
20000
15000
10000
5000
0
pHGT4-2031
pHGT4d-828
pHGT4d-313
pHGT4d-119
pHGT4d-92
pHGT4d-41
Reporter constructs
C.
-313
-134
-35
Luc
mock
SREBP-1c
Fold Increase
8
7
6
5
4
3
2
1
0
pHGT4d-313
PHGT4t-215
Reporter construct

Figure 4. Effects of SREBP-1c on human GLUT4 promoter (A) Deletion constructs of human GLUT4 promoter **(B)** Promoter activities of deletion constructs. Cells were cotransfected with 0.5 μg of pHGT4-serial deletion constructs with 0.1 μg of pSV_SPORT expression vector for SREBP-1c (black bars) or of pSV_SPORT alone (white bars). The luciferase activities were assayed 48 h after transfection. The reporter activities were shown as the relative luciferase activities normalized with protein concentration **(C)** Promoter activities of truncation construct in 3T3-L1 preadipocytes. Normalized luciferase activities were expressed as fold increase relative to the basal activity respectively in the absence of the SREBP-1c expression vectors. The data represent the mean ± S.D. of three independent experiments performed in triplication.

5. SREBP-1c binds to the human GLUT4-SRE

To determine whether SREBP-1c activates human GLUT4 promoter through direct DNA binding, we performed EMSA using GLUT4-SREa (the region from -114 to -70) as a probe. Fig. 4 shows that the SRE probe corresponding to the SRE in the human GLUT4 promoter was shifted and increased after the addition of the SREBP-1 protein in dose-dependent manner (lane 2, 3, 4, and 5). The specificity of the binding was conformed by its supershift by SREBP-1 antibody (lane 7). In competition assays, the shifted band disappeared upon the addition of an excess amount of the unlabeled SREa probe (lane 6). These data are consistent with the results of the luciferase assays and indicate that SREBP-1c activates the human GLUT4 promoter through binding to the SRE (SRE1 and/or SRE2).

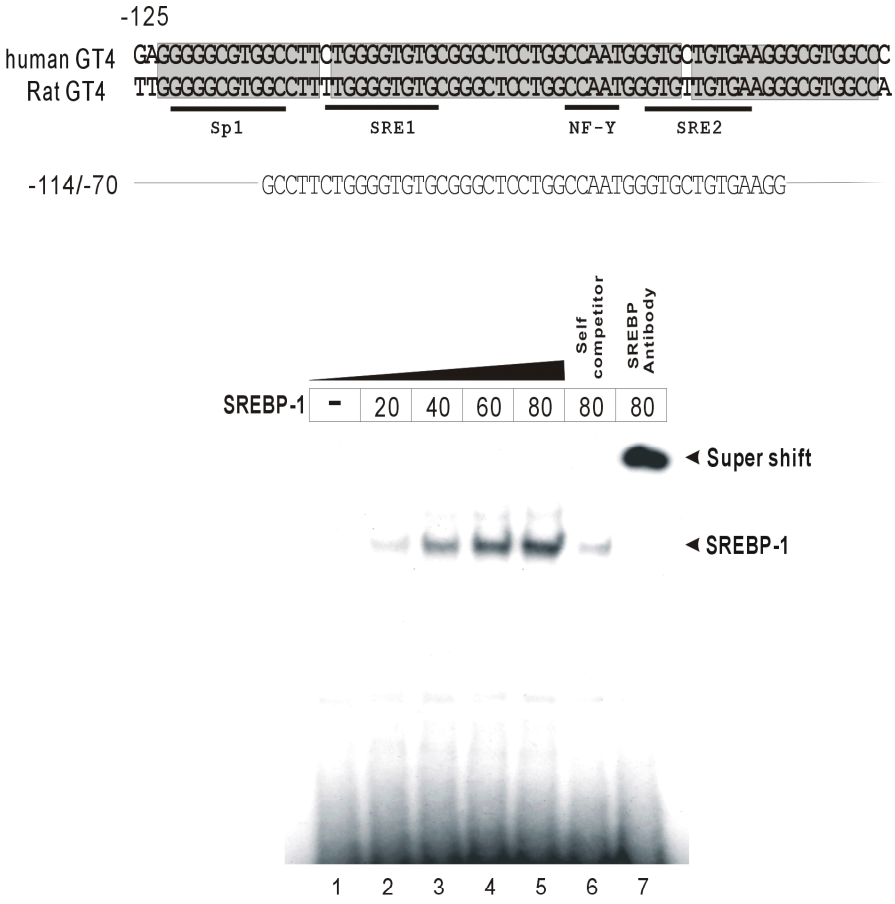
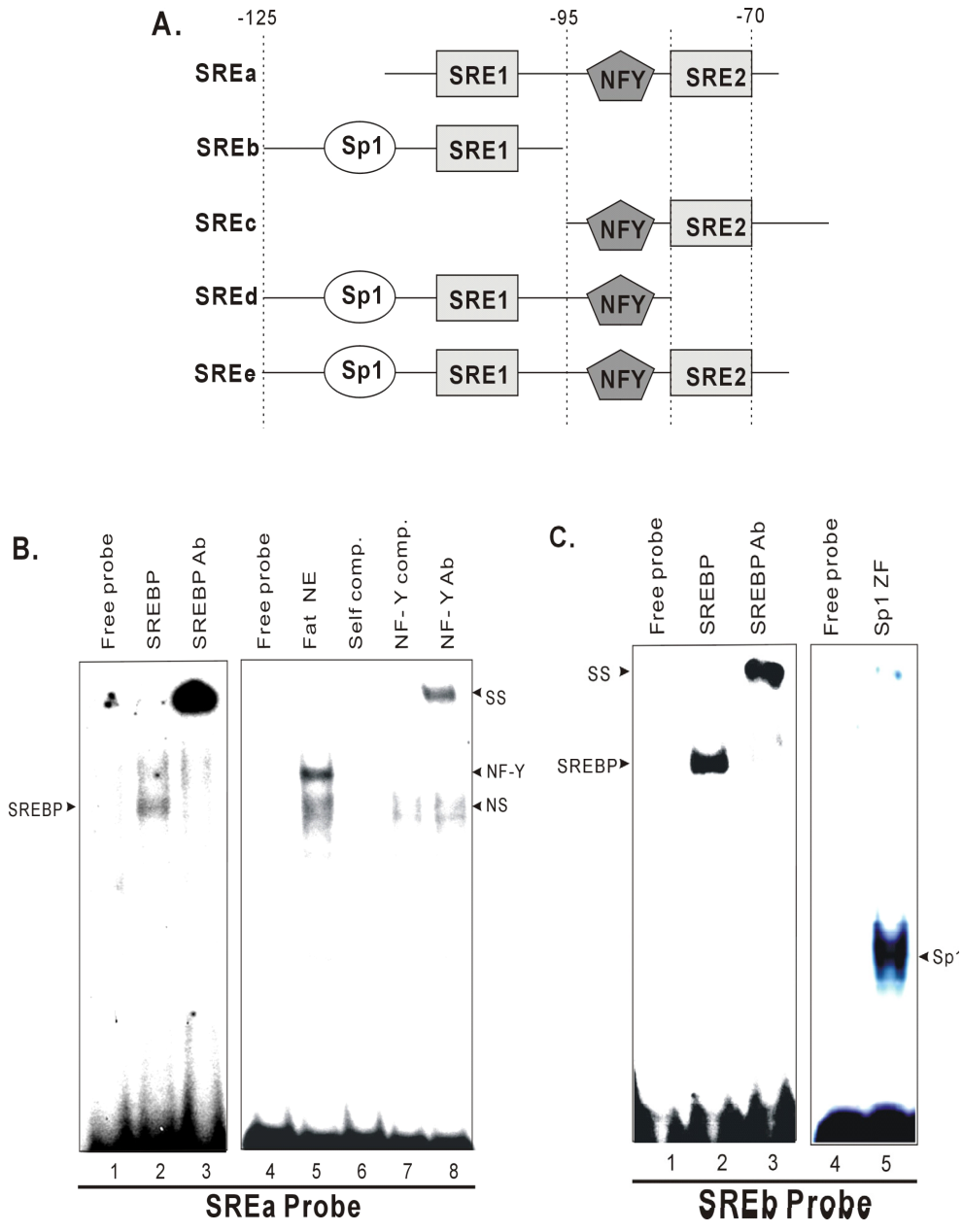
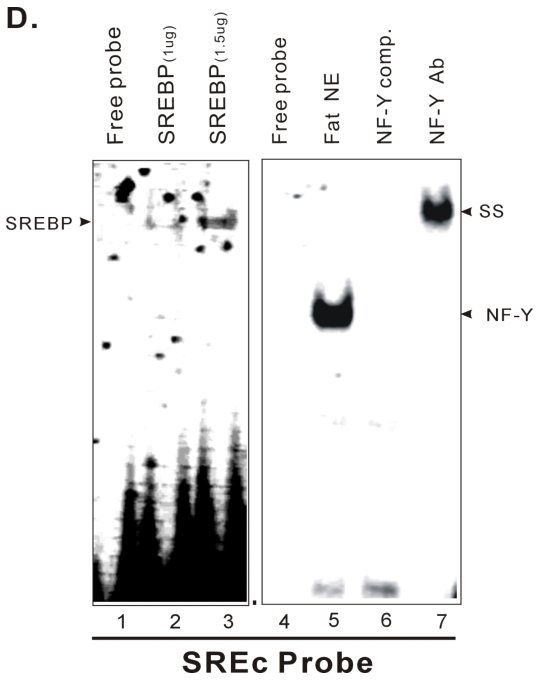


Figure 5. SREBP-1 binds to the putative SRE sequence in the GLUT4 promoter. The probe was incubated in the reaction mixture with (lanes 2-7) or without (lane 1) recombinant SREBP-1 protein for 30 min on ice. Specificity of SREBP-1 binding (the upper arrow) was confirmed by a supershift after the addition of SREBP-1 antibody (lane 7).

6. The binding of Sp1, SREBP-1, and NF-Y to GLUT4 promoter

To demonstrate the direct binding of Sp1, SREBP-1, and NF-Y to the human GLUT4 promoter, gel mobility shift assay was performed (Fig 6A). SREa probe was shifted after the addition of 100 ng SREBP-1 protein (Fig. 5 and Fig 6B. left), and 1 μg fat nuclear extract (Fig 6B. right). The bind was supershifted by SREBP-1 antibody (Fig 6B. lane3), and NF-Y antibody (Fig 6B. lane8). In competition assays, the shifted band was disappeared by addition of excess amount of the cold SREa probe (Fig 6B. lane6) or NF-Y probe (Fig 6B. lane7). To identify putative SRE1 and SRE2 to in the GLUT4 promoter, EMSA was performed using SREb (including Sp1, SRE1) and SREc (including NF-Y, SRE2) probes. Fig. 5C shows the binding of SREBP-1 and Sp1. The binding of SREBP-1 to the SREc was very weak although NF-Y was bound (Fig. 6D, left). These results suggest that SREBP-1 activates the human GLUT4 promoter through binding to the SRE1 rather than SRE2. Fig. 6E and 6F show that the SREd and SREe probe were shifted after the addition of recombinant SREBP-1 or Sp1 protein. We cannot observe the binding of NF-Y although SREd and SREe probes contains the NF-Y-binding site. These data indicate that SREBP-1c activates the human GLUT4 promoter mainly through binding to the SRE1 but not through SRE2. Moreover, Sp1 binding to the human GLUT4 promoter eliminates NF-Y binding to GLUT4 promoter.


A.
-125
-95
-70
SREa
SRE1
NFY
SRE2
SREb
Sp1
SRE1
SREc
NFY
SRE2
SREd
Sp1
SRE1
NFY
SREe
Sp1
SRE1
NFY
SRE2
B.
Free probe
SREBP
SREBP Ab
Free probe
Fat NE
Self comp.
NF-Y comp.
NF-Y Ab
SS
NF-Y
NS
SREBP
1 2 3 4 5 6 7 8
SREa Probe
C.
Free probe
SREBP
SREBP Ab
Free probe
Sp1 ZF
SS
SREBP
Sp
1 2 3 4 5
SREb Probe


D.
Free probe
SREBP(1ug)
SREBP(1.5ug)
Free probe
Fat NE
NF-Y comp.
NF-Y Ab
SREBP
SS
NF-Y
1 2 3 4 5 6 7
SREc Probe

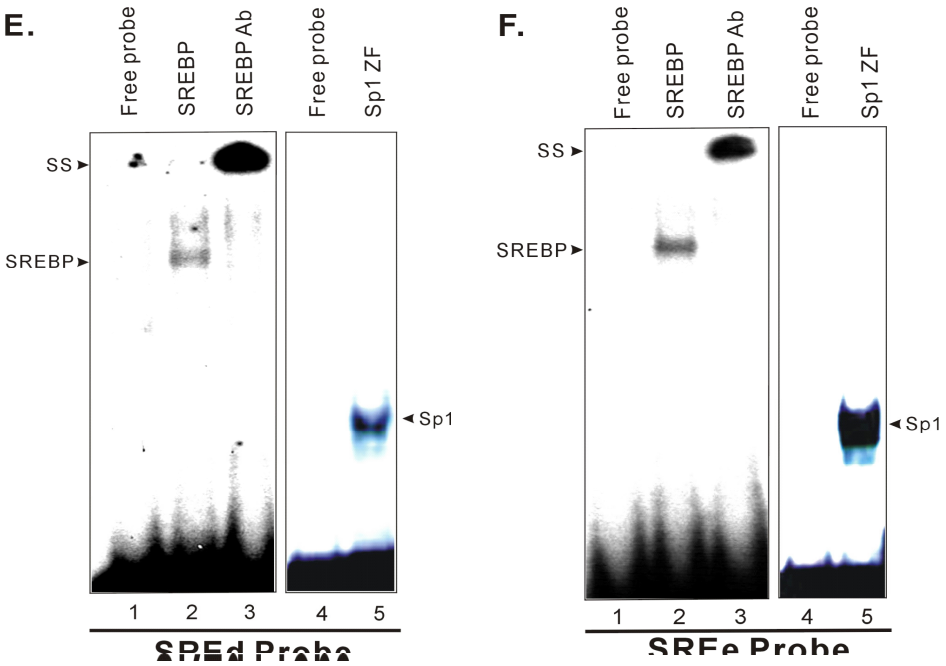

E.
Free probe
SREBP
SREBP Ab
Free probe
Sp1 ZF
SS
SREBP
Sp1
1 2 3 4 5
F.
Free probe
SREBP
SREBP Ab
Free probe
Sp1 ZF
SS
SREBP
Sp1
1 2 3 4 5

Figure 6. EMSA of human GLUT4 promoter (A) Schematic representation of probes used for EMSA. The sequences were shown in Table 1. **(B)-(F)** Probes (50,000 cpm of [γ-^{32}P]ATP-labeled human GLUT4 promoter) were incubated in the reaction mixture with 120ng recombinant SREBP-1 protein (except fig 6D), 100ng recombinant Sp1 zinc finger protein, or 1 μg nuclear extract for 30 min on ice. Specificity of SREBP-1 and NF-Y binding was confirmed by supershift assay.

Table 2. Binding of Sp1, SREBP-1, and NF-Y according to the oligonucleotides

	Sp1 binding	SREBP-1 binding	NF-Y binding
SREa	X	O	O
SREb	O	O	Not tested
SREc	X	O	O
SREd	O	O	X
SREe	O	O	X

O : binding, X : not binding

7. Effects of mutations in the Sp1, SRE1, and NF-Y binding region of the human GLUT4 promoter.

In order to know the effects of mutation in the Sp1, SRE1, and NF-Y binding region on the human GLUT4 promoter activity (Fig. 7A), we constructed mutant versions for each consensus sequence and measured their promoter activities to evaluate their contributions (Fig. 7B). Sp1 (Ma) mutation of the pHGT4d-313 inhibited SREBP-1c driven promoter activity by 66%. The mutation of SRE1 (Mb) resulted in 68% loss of SREBP-1c dependent activation and the mutation of NF-Y (Mc) resulted in 48% loss of activation. SREBP-1c could not activate the promoter containing double mutations of SRE1 and NF-Y (Mbc). Also, mutation of Sp1 and NF-Y (Mac) resulted in 75% loss of activation. Interestingly, SREBP-1c activated the promoter activity when Sp1 and SRE1 (Mab) sites were mutated simultaneously when compared to Ma or Mb. Taken together, it is suggested that SRE1 and NF-Y play a critical role in SREBP-1c-mediated induction of human GLUT4 promoter. However, the functional relationship between Sp1, SRE1, and NF-Y needs to be further investigated.

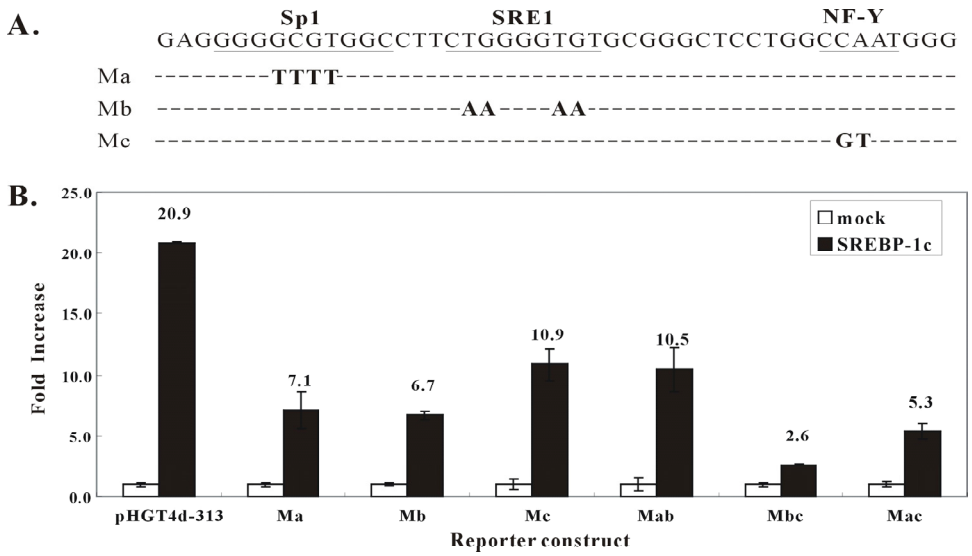


Figure 7. Effects of mutations on Sp1, SRE, and NF-Y binding site on SREBP-1c-mediated activation of human GLUT4 promoter (A) Structure of wild type and mutants of putative Sp1, SRE1, and NF-Y consensus sequence (B) Wild or mutant type human GLUT4 promoter luciferase reporter constructs were transfected into 3T3-L1 preadipocytes with (black bar) or without (white bar) overexpression of SREBP-1c. Mutant constructs pHGT4-Sp1m (Ma), pHGT4-SREm (Mb), pHGT4-NF-Ym (Mc), pHGT4-Sp1m/SREm (Mab), pHGT4-SREm/NF-Ym (Mbc) ,and pHGT4-Sp1m/NF-Ym (Mac) were produced by substitution mutations into pHGT4d-313 using site-directed mutagenesis. Normalized luciferase activities were expressed as fold increase relative to the basal activity respectively in the absence of the SREBP-1c expression vectors.

IV. DISCUSSION

Studies on glucose transporter have focused predominantly on the insulin-regulated glucose transporter translocation in adipocyte or muscle. The GLUT4 translocation by insulin is one of the control mechanism of whole body glucose homeostasis. Dysregulation of this mechanism is one of the key phenomenon in the pathogenesis of type 2 diabetes mellitus. Expression of GLUT4 is altered in response to changing metabolic conditions and insulin. A high-fat diet leads to reduction in GLUT4 levels in both skeletal muscle and adipose tissue.[23] In rats, GLUT4 expression is known to be reduced in adipose tissue, but increased in skeletal muscle during fasting.[24]

Recently, novel transcriptional SREBP-1c target genes were identified. FAS, LDL receptor, HMG-CoA reductase, high density lipoprotein receptor SR-BI, SCD-1, GAPDH, GLUT1, PAI-1, and the β and δ isoforms of C/EBP exhibited coordinate induction with insulin and SREBP-1c overexpression.[22] The transcriptional upregulation of FAS[27], GAPDH,[28] GLUT1,[29] SCD-1,[30] PAI-1,[31] and C/EBPβ and -δ[32] by insulin has been demonstrated in adipocytes. Five of the insulin-regulated genes, FAS,[33] SCD-1,[34] LDL receptor,[35] HMG-CoA reductase,[36] and SR-BI,[47] have been previously characterized as direct SREBP targets, indicating the importance of SREBP-1c for insulin effects on gene expression.

In the present study, we identified two SREBP-1c response elements, SRE1 and SRE2, in human GLUT4 promoter. SREBP-1c could bind to these SREs and activate the human GLUT4 promoter. EMSA study showed that SRE1 has stronger affinity to SREBP-1 than SRE2. Sp1 and NF-Y also bind to the human GLUT4 promoter, respectively. Interestingly, when Sp1 binds to the Sp1-binding site of human GLUT4 promoter, NF-Y does not. However, transient transfection assay showed that all of the Sp1, SRE, and NF-Y were necessary for the transactivation of human GLUT4 promoter by SREBP-1c.

At present, the molecular interactions between three transactivators affecting the GLUT4 promoter activity are not known. Detailed studies are underway to dissect the contribution of these transcriptional activators to the GLUT4 gene.

V. CONCLUSION

1. The expression of human GLUT4 and SREBP-1c are controlled at transcriptional level by food intake in adipose tissue.
2. Human GLUT4 promoter is stimulated by overexpression of SREBP-1c in 3T3-L1 preadipocyte.
3. The region from -119 to -41 plays an important role in mediating responsiveness of human GLUT4 promoter to SREBP-1c.
4. SREBP-1 have the strong affinity to SRE1 (-109/-101) and the weak affinity to SRE2 (-81/-73).
5. Sp1 and NF-Y bind to the -122/-113 and -88/-84 region of human GLUT4 promoter, respectively. However, when Sp1 binds to the human GLUT4 promoter, NF-Y doesn't.

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Abstract (in Korean)

지방 조직에서 제 4 형 포도당 운반체의 전사 조절 기전

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강승연

제 4 형 포도당 운반체는 근육과 지방 조직에서 주로 발현되며 인슐린에 의해 조절된다. 제 4 형 포도당 운반체는 생체 내에서 당질의 항상성을 유지하는데 중요한 역할을 한다고 알려져 있으나, 제 4 형 포도당 운반체 유전자 발현의 전사 조절과 관련된 연구는 아직 미흡한 상태이다. 따라서 본 연구에서는 식이 시 지방 조직에서 인슐린의 조절을 받는 전사 인자인 SREBP-1c 가 제 4 형 포도당 운반체의 유전자 조절에 어떤 영향을 미치는지 조사하였다.

백서의 지방에서 제 4 형 포도당 운반체와 SREBP-1c 의 발현은 식이 시 유전자 전사 수중에서 크게 증가되었다. 또한 인슐린과 식이 조절에 따라 유전자 전사를 조절하는 전사 인자인 SREBP-1c 는 제 4 형 포도당 운반체 promoter 의 -119/-41 부위 내에 작용하여 발현을 조절함을 알았다. EMSA 를 수행한 결과 두 개의 SRE (-109/-101 그리고 -81/-

73), Sp1 결합 부위 (-122/-113), 그리고 NF-Y 결합 부위 (-88/-84)에 각각 SREBP, Sp1, 그리고 NF-Y 가 결합함을 확인하였다. Sp1 결합 부위, SRE1, 그리고 NF-Y 결합 부위를 돌연변이 시켰을 때 SREBP-1c 에 의한 promoter 활성이 감소하였다. 이상의 결과를 통해서 지방 조직에서 제 4 형 포도당 운반체의 유전자 발현이 식이 조절에 따라 전사 수준에서 조절되고 이는 SREBP-1c 를 통해 이루어짐을 알 수 있었다. 또한 SREBP-1c 에 의한 제 4 형 포도당 운반체 유전자의 발현 증가에 Sp1 과 NF-Y 가 중요한 역할을 할 것으로 사료된다.

핵심되는 말 : 제 4 형 포도당 운반체, 지방 조직, Sterol regulatory element-binding protein-1c (SREBP-1c), 전사 조절 , Sp1, NF-Y