Genes: Structure and Regulation:

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The regulation of hepatic glucose metabolism is important in glucose homeostasis, and liver glucokinase (LGK) plays a central role in this process. Hepatic glucokinase expression is known to be regulated by insulin. Recently it has been suggested that sterol regulatory element binding protein-1c (SREBP-1c) mediates the action of insulin on LGK transcription; however, the precise mechanism is not, to date, well known. In the present study, we identified two functional SREBP-1c response elements, SREa and SREb, in the rat LGK promoter. SREBP-1c could bind to these SREs and activate the LGK promoter, and insulin activated the LGK promoter in Alexander cells. The physical interaction between the protein and SREs of the LGK promoter *in vivo* was also confirmed. Insulin selectively increased SREBP-1c and LGK expression in primary hepatocytes. Adenoviral expression of SREBP-1c stimulated LGK expression, and the dominant negative mutant of SREBP-1c blocked the increased gene expression of LGK by insulin and SREBP-1c. A chromatin immunoprecipitation assay using primary hepatocytes showed increased binding of SREBP-1 to SREs of the LGK promoter by insulin.

Liver is important for glucose and lipid metabolism, and hepatic glucose and lipid metabolism are known to be mainly regulated by insulin. The typical action of insulin in liver is to increase glycolysis, glycogenesis, and lipogenesis. When glucose is delivered to the liver in large quantities it is converted to glycogen and stored. If the hepatic glycogen stores are repleted, glucose enters the glycolysis pathway to produce pyruvate for *de novo* lipogenesis, and the lipid is exported as very low density lipoprotein and stored as triacylglycerol in adipose tissue. Thus, the regulation of hepatic glucose metabolism is important in glucose homeostasis.

Glucose is phosphorylated by glucokinase (GK)[1] (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1) to produce glucose-6-phosphate, which enters the glycolysis and glycogenesis pathway (1). Because the K_m of GK is considerably higher than that of normal blood concentrations and because GK is not subjected to allosteric regulation by the product, the rate of glucose phosphorylation is proportional to the blood glucose level. Therefore, GK is thought to be essential for the liver in sensing glucose and maintaining the metabolic function (2, 3).

GK is mainly expressed in liver, pancreatic β cells, and neuroendocrine cells of brain and gut. Two alternate promoters regulate the tissue-specific expression of GK in liver and pancreatic β cells (4). The upstream promoter regulates β cell-specific GK expression, and the downstream promoter controls liver-specific GK (LGK) expression. In the liver, GK expression is regulated in response to fasting and refeeding (5), with insulin and glucagon serving as the mediators of this response. Hepatic GK expression is reduced in the diabetic animal models with insulin deficiency and insulin resistance (6, 7). Insulin stimulates GK expression in primary hepatocytes regardless of glucose concentration, and glucagon inhibits GK expression (8, 9). These results indicate that insulin is a major activator of GK transcription. However, the *cis*-element in the LGK promoter that is responsible for insulin responsiveness has not yet been identified. Besides transcriptional regulation, hepatic GK activity can be regulated by the GK regulatory protein. At low glucose concentrations, the GK regulatory protein is associated with GK in the nucleus (10). Acute glucose challenge induces dissociation of the GK-GK regulatory protein complex, and GK is then translocated into cytoplasm, resulting in increased GK activity (11, 12).

Sterol regulatory element-binding protein-1c (SREBP-1c), also known as adipocyte determination and differentiation dependent factor 1 (ADD1), is a member of the SREBP family that regulates the transcription of genes involved in cholesterol and fatty acid synthesis (13, 14). SREBPs are synthesized in a precursor form bound to the endoplasmic reticulum and the nuclear membrane. After proteolytic cleavage, mature SREBPs enter the nucleus and activate the transcription of the target genes by binding to sterol response element (SRE) or E-box-like sequences. Three isoforms of SREBPs from two genes have been identified. SREBP-1a and −1c are encoded from a single gene but are different in their first exon due to alternative usage of the promoter. Because two isoforms of SREBP-1 have the same DNA binding domain, they recognize the same DNA element. However, transcriptional activity of SREBP-1a is stronger than that of SREBP-1c, because SREBP-1a has a longer acidic transactivation domain (15). SREBP-1a is a potent activator of all SREBP-responsive genes, whereas the roles of SREBP-1c and SREBP-2 are more restricted in that

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[1] The abbreviations used are: GK, glucokinase; SRE, sterol regulatory element; SREBP, SRE-binding protein; ChIP assay, chromatin immunoprecipitation assay; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay.

SREBP-1c preferentially activates genes required for fatty acid synthesis, and SREBP-2 activates genes for cholesterol synthesis. Among three isoforms of SREBPs, SREBP-1c is mainly expressed in liver and is regulated by nutritional status. Recently, SREBP-1c was suggested to be a transcription factor that mediates the action of insulin on GK transcription in liver (23, 24). However, the mechanism used by SREBP-1c to induce hepatic GK expression in response to insulin is not well characterized.

Here, we identify SREs in the rat LGK promoter and present evidence that insulin increases the binding of SREBP-1c on the LGK promoter resulting in the increase of the LGK transcription. These results indicate that SREBP-1c is a transcription factor mediating the effects of insulin on the regulation of LGK gene expression.

EXPERIMENTAL PROCEDURES

Plasmids—The luciferase reporter construct under the control of the rat LGK promoter pRGL-1448 was described earlier (16). pRGL-593, pRGL-238, pRGL-161, pRGL-76, pRGL+4, and pRGL+55 were constructed by amplifying the rat LGK promoter regions of −593/+127, −238/+127, −161/+127, −76/+127, +4/+127, and +55/+127 and subcloning them into pGL3 basic vector, respectively. pRGL-1448ma, pRGL-1448mb, and pRGL-1448mab were produced by introducing a substitution mutation into pRGL-1448 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of all constructs were confirmed by DNA sequencing. The SRE-truncated mutant pRGLΔab, the −210/-167 region of which was truncated from pRGL-1448, was constructed by PCR using a SRE-truncation primer (sense, 5′-TTCTTTAACCAGTCTCTCTCCACAGCAAGT-3′; antisense, 5′-ACTTGCTGTGGAGAGAGACTGGTTAAAGAA-3′). The expression plasmid of SREBP-2 (pCS2) and the preparation of recombinant SREBP-1 were described previously (17). Expression plasmids of SREBP-1c and the dominant negative mutant of SREBP-1c (SREBP-1cDN) were a kind gift from Drs. B. M. Spiegelman and J. B. Kim (18). We subcloned SREBP-1c into pcDNA3 for experimental purposes.

Animals and Diet—Male Sprague-Dawley rats (150–200 g) were used for the experiments. Rats were fasted for 24 h and refed a fat-free high carbohydrate diet containing 82% (w/w) carbohydrates (74% starch, 8% sucrose), 18% (w/w) casein, 1% vitamin mix, and 4% (w/w) mineral mix. All components for the diet were purchased from Harlan Teklad Co. (Madison, WI).

Cell Culture and Transient Transfection Assay—Alexander cells (American Type Culture Collection number CRL-8024) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfection and luciferase assays were performed as described previously (19). Briefly, Alexander cells were plated at a density of 2×10^5 cells/35-mm dish. On the following day, 500 ng of luciferase reporter plasmid and 50 ng of overexpression plasmid were transfected using LipofectAMINE Plus reagent (Invitrogen). To transfect a constant amount of DNA, sample DNAs were supplemented with control vector pcDNA3. Protein concentration was determined by the method of Bradford. Luciferase activities were normalized by protein concentration to adjust transfection efficiency. Normalized luciferase activities were shown as mean ± S.D. of three independent experiments in triplicate and were expressed as fold increase relative to the basal activity of the reporters in the absence of overexpression vectors.

Isolation of Total RNA, Northern Blot Analysis, and Reverse Transcription PCR—Total RNA was extracted from liver and primary hepatocytes using TRIzol reagent (Invitrogen) according to the manufacturer's protocol (19). Twenty micrograms of total RNA was separated on 1% agarose gels containing 0.66 M formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane by capillary transfer in 20× SSC and UV-crosslinked. The 500-bp fragment representing exon 2 to exon 4 of the rat GK gene, the 500-bp fragment of exon 2 to exon 4 of the rat SREBP-1 gene, and the 500-bp fragment of exon 2 to exon 4 of the rat SREBP-2 gene were labeled with [α-^{32}P]dCTP using the Rediprime labeling kit (Amersham Biosciences) and used as cDNA probes. The membrane was hybridized with ^{32}P-labeled cDNA probes in Rapid-hyb buffer (Amersham Biosciences) at 65 °C for 4 h. After hybridization, the membrane was washed with 2× SSC 0.1% SDS followed by 0.2× SSC 0.1% SDS and exposed to x-ray film at −70 °C with an intensifying screen.

For reverse transcription PCR, first strand cDNA was synthesized from 4 μg of total RNA in a 20-μl volume using random hexamer and Superscript II reverse transcriptase (Invitrogen). One microliter of the reverse transcription reaction mixture was amplified with primers specific for rat LGK and β-actin in a total volume of 50 μl. Linearity of the PCR was tested by amplifying 100 ng of total RNA in the amplification cycles between 20 and 50. According to the amplification profile, samples were amplified for 30 cycles using the parameters 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. β-actin was used as an internal control for the quality and quantity of RNA. The identity of PCR products were subjected to electrophoresis on 1.5% agarose gel, and the quantities of PCR products were analyzed by Molecular Analyst II (Bio-Rad). The PCR product was confirmed by DNA sequencing. Primers used in PCR were as follows: LGK-sense, 5′-CAGACAGTCCTCACCTGCAA-3′; LGK-antisense, 5′-TGACCAGCATCACTCTGAAG-3′; SREBP-1a-sense, 5′-ACACAGCGGTTTTGAACGACATC-3′; SREBP-1a-antisense, 5′-ACGGACGGGTACATCTTTACAG-3′; SREBP-1c-sense, 5′-GGAGCCATGGATTGCACATT-3′; SREBP-1c-antisense, 5′-AGGAAGGCTTCCAGAGAGGA-3′; β-actin-sense, 5′-TTGTAACCAACTGGGACGATATGG-3′; and β-actin-antisense, 5′-CGACCAGAGGCATACAGGGACAAC-3′.

DNase I Footprinting Assay—A DNase footprinting assay was performed as described previously (20). DNA fragments covering from −320 to −69 of the LGK gene were labeled in sense strand and purified as follows. The promoter region was amplified by PCR, and the resulting fragments were subcloned into the KpnI and EcoRI sites of the pBluescript SK+ vector. The promoter fragments were isolated by double digestion with KpnI and EcoRI to obtain 5′-overhanging ends and 3′-overhanging ends. The fragments were labeled using a Klenow fragment and [α-^{32}P]dATP and then purified from PAGE. DNA protein binding reactions were performed using 50,000 cpm (~1 ng) of probe per reaction in a solution containing 10 mM HEPES, pH 7.9, 60 mM KCl, 7% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC), and the indicated amount of purified recombinant SREBP-1. After a 20-min 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% glycerol was added to the reaction and kept at room temperature for 2 min. Dilution folds of DNase I were ranged from 1:200 to 1:2000 of stock (10 units/μl), depending on the amount of protein in the reaction. Digestion reactions were stopped by adding 80 μl of a stop solution containing 20 mM Tris-Cl, pH 8, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of yeast tRNA, and 10 μg of proteinase K. The samples were resolved in 6% polyacrylamide and 7 M urea sequencing gel. The protected regions were mapped with reference to the migration of Maxam-Gilbert sequencing products.

Electrophoretic Mobility Shift Assay (EMSA)—The oligonucleotide probes P-216/-187 (for SREa), P-216/-187ma, P-188/-159 (for SREb), and P-188/-159mb were labeled as described previously (19). Ten picomoles of single-stranded sense oligonucleotide were labeled with ^{32}P using T4 polynucleotide kinase (TaKaRa, Shiga, Japan) and annealed with a 5 M excess of antisense oligonucleotide. ^{32}P-labeled, double-stranded oligonucleotides were purified with Sephadex G50 (Amersham Biosciences) spin columns. P-225/-150, P-225/-150ma, P-225/-150mb, and P-225/-150mab were generated by PCR using pRGL-1448, pRGL-1448ma, pRGL-1448mb, and pRGL-1448mab as templates. ^{32}P-labeled sense oligonucleotides (5′-TTCTTTAACCAGTCTGAAGG-3′) and unlabeled antisense oligonucleotides (5′-GGACTTGCTGTGGAGAGGTT-3′) were used to amplify the probes. The labeled probes were purified by PAGE. 50,000 cpm (~0.02 pmol) of probes were incubated with a recombinant SREBP-1 protein for 20 min on ice in a buffer containing 10 mM HEPES (pH 7.9), 60 mM KCl, 10% glycerol (v/v), and 1 mM dithiothreitol. One μg of poly(dI-dC) was added to each reaction to suppress nonspecific binding. For competition assays, excessive unlabeled oligonucleotides were added to the reaction mixture. Two microliters of anti-SREBP-1 serum was added to the reaction for a supershift assay. The protein-DNA complexes were resolved from free probe by PAGE at 4 °C on a 4% polyacrylamide gel in 0.5 × Tris borate-EDTA buffer. The dried gels were exposed to x-ray film at −70 °C with an intensifying screen.

Chromatin Immunoprecipitation (ChIP) Assay—Male Sprague-Dawley rats (~150 g) had free access to Purina chow and were divided into fasting and refeeding groups. The fasting group fasted for 24 h. The refeeding group had free access to high carbohydrate diet for 24 h after fasting. The liver was perfused with serum-free DMEM for 5 min and cross-linked with 5% formaldehyde in serum-free DMEM for 5 min. The livers were homogenized, pelleted by centrifugation at 2000 rpm for 4 min at 4 °C, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8). The lysate was sonicated on ice for 3 min with a 2.5 mm in diameter sonicator tip at 30% amplitude and a 0.5 cycle (UP400S; Dr. Hielscher GmbH, Teltow, Germany) and sheared to

100–600 bp. To provide a positive control (input) for each condition, one undiluted aliquot was retained for further processing in parallel with all other samples at the reversal of the cross-linking step. To reduce nonspecific background, each chromatin sample (1 ml) was precleared using 60 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA), supplemented with 200 μg/ml sonicated salmon sperm DNA (Stratagene), and the beads were pelleted. Chromatin complexes in the supernatant were immunoprecipitated overnight at 4 °C using 30 μg of anti-SREBP-1 serum or preimmune serum or without serum. Immune complexes were collected with 60 μl of protein A/G-agarose and 200μg/ml salmon sperm DNA. Chromatin complexes were eluted from the beads and used for subsequent PCR analysis. LGK promoter-specific primers were 5′-ATCCCACGAGGTACCCCCACTATTC-3′ (sense) and 5′-GACTTGCTGTGGAGAGGTTA-3′ (antisense).

Preparation of Recombinant Adenovirus—The cDNA encoding SREBP-1c or SREBP-1cDN was cloned into the pAd-YC2 shuttle vector, which has the cytomegalovirus promoter and multiple cloning sites, including the bGHp(A) sequence (21). For homologous recombination, a shuttle vector, pAd-YC2 (5 μg), and a rescue vector, pJM17 (5 μg), were cotransfected into 293 cells, which had been cultured in a 24-well plate the day before transfection. After 12–15 days, recombinants were screened by PCR using upstream primers derived from the cytomegalovirus promoter and downstream primers from the bGHp(A) sequence. Then, the recombinants were amplified in 293 cells and purified and isolated using $CsCl_2$ (Sigma). The preparations were collected and desalted, and titers were determined by the measurement of plaque counts.

Isolation of Primary Hepatocytes and Treatment of Recombinant Adenovirus—Primary hepatocytes were isolated from normal Sprague-Dawley rats (~150 g) as described previously (22). The primary hepatocytes were incubated for 2 h with an adenovirus containing DMEM at a titer of 15 plaque-forming units per cell for 2 h at 37 °C. Then, culture medium was replaced by DMEM supplemented with 10% fetal calf serum. The infected cells were harvested for RNA isolation 24 h after viral infection.

Statistical Analysis—All transfection studies were performed in triplicates and repeated 3–5 times. The data were represented as mean ± S.D. Statistical analysis was carried out using Microsoft Excel (Microsoft, Redmond, WA).

RESULTS

SREBP-1c Activates LGK Promoter—To determine the response of the LGK promoter to SREBPs, a luciferase reporter construct under the control of the LGK promoter pRGL-1448, which contained the −1448/+127 region of the rat LGK gene, was prepared and transfected into Alexander cells with or without expression of SREBPs. As shown in Fig. 1, SREBP-1c activated the LGK promoter, whereas SREBP-2 did not. We also observed that SREBP-1a activated the promoter construct (data not shown). This result suggested the presence of SRE in the LGK promoter and the preference of the SRE to SREBP-1, which was observed in many promoters of many lipogenic genes.

Localization of SRE in the LGK Promoter—To localize the region responsible for the activation by SREBP-1, we prepared 5′ deletion constructs and tested their responsiveness to SREBP-1c in Alexander cells. As shown in Fig. 2*A*, pRGL-1448, pRGL-593, and pRGL-238 were activated by SREBP-1c. But pRGL-161 was slightly activated by SREBP-1c. This sight activation did not disappear even in the reporter without promoter. This marginal activation of reporter without promoter might be due to the presence of an E-box in front of the multiple cloning site of the pGL3basic plasmid. Thus, we assumed that −238/−161 region was necessary for the activation of the LGK promoter by SREBP-1c. To localize SREBP-1c binding sites in the LGK promoter, a DNase I footprinting assay was performed using a recombinant human SREBP-1 protein (17). As shown in Fig. 2*B*, the region from −210 to −167 of the LGK promoter was protected from DNase I digestion by SREBP-1 protein, which suggested the binding of SREBP-1 to this region. However, the protected region was wider than expected, and sequence analysis revealed the presence of two SRE consensus sequences (YCAYNYCAY) in the −210/−167 region. An EMSA was performed using the probe P-225/-150, which covered the

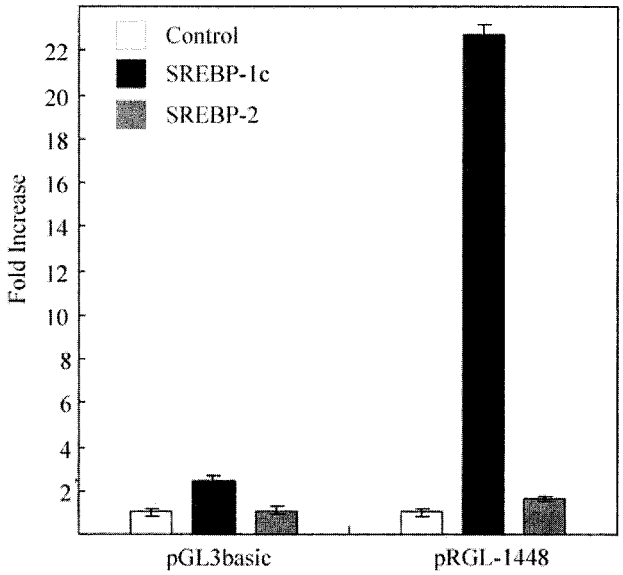

FIG. 1. **SREBP-1c activates LGK promoter.** Luciferase reporter constructs under the control of the LGK promoter spanning −1448 to +127, pRGL-1448, and the control pGL3basic vector were transfected into Alexander cells. Expression plasmids of SREBP-1c (*black bars*) or SREBP-2 (*gray bars*) or the control plasmid pcDNA3 (*white bars*) was cotransfected with the reporter. Normalized luciferase activities were expressed as fold increase relative to the basal activity in the absence of the SREBP-1c expression vector and represented mean values ± S.D. from three independent experiments.

−225/−150 region of LGK promoter, to confirm the binding of SREBP-1 to the region. Fig. 2*C* shows two shifted bands by SREBP-1. Because recombinant SREBP-1 protein was used in EMSA, these results suggested that two distinct and closely located SREBP-1 binding sites exist in the −210/−167 region.

Identification of Two Distinct SREs in the LGK Promoter—We designated two putative SREs as SREa and SREb, respectively, and produced probes P-216/-187 and P-188/-159, which contained SREa and SREb, respectively (Fig. 3*A*). An EMSA using P-216/-187 and P-188/-159 probes showed the binding of SREBP-1 to each SRE (Fig. 3*B*). But SREBP-1 could not bind to mutant probes P-216/-187ma and P-188/-159mb, which had mutations in SREa and SREb, respectively. In addition, we observed that the intensity of the shifted band of P-216/-187 was stronger than that of P-188/-159. To test the specificity of SREBP-1 binding, we performed competition assay and supershift assay using P-216/-187 (Fig. 3*C*). We employed P-216/-187, P-188/-159, P-216/-187ma, and P-188/-159mb as unlabeled competitors. An unlabeled self-competitor, P-216/-187, could completely compete the binding of SREBP-1 to the probe P-216/-187 (Fig. 3*C*, *lanes 3* and *4*). However, unlabeled P-188/-159 competed the probe P-216/-187 less efficiently than did the self-competitor (Fig. 3*C*, *lanes 5* and *6*). SRE-mutated P-216/-187ma and P-188/-159mb did not compete the probe (Fig. 3*C*, *lanes 7–10*). In addition, the anti-SREBP-1 antibody completely blocked the binding of SREBP-1 to the probe (Fig. 3*C*, *lane 12*). We also performed the same experiment using P-188/-159 to confirm the specificity of SREBP-1 binding to SREb (data not shown). From these results, it was concluded that SREBP-1 could bind to both SREa and SREb and that the binding affinity of SREa was stronger than that of SREb.

To know the functional property of the two SREs, we constructed mutant version of promoter-luciferase reporter constructs and observed the response to SREBP-1c (Fig. 4*A*). The internal deletion of the −210/−167 region from the LGK promoter (pRGL-1448Δab) resulted in the complete loss of activation by SREBP-1c. Based on an EMSA experiment, we introduced mutations of two nucleotides into SREa and/or SREb

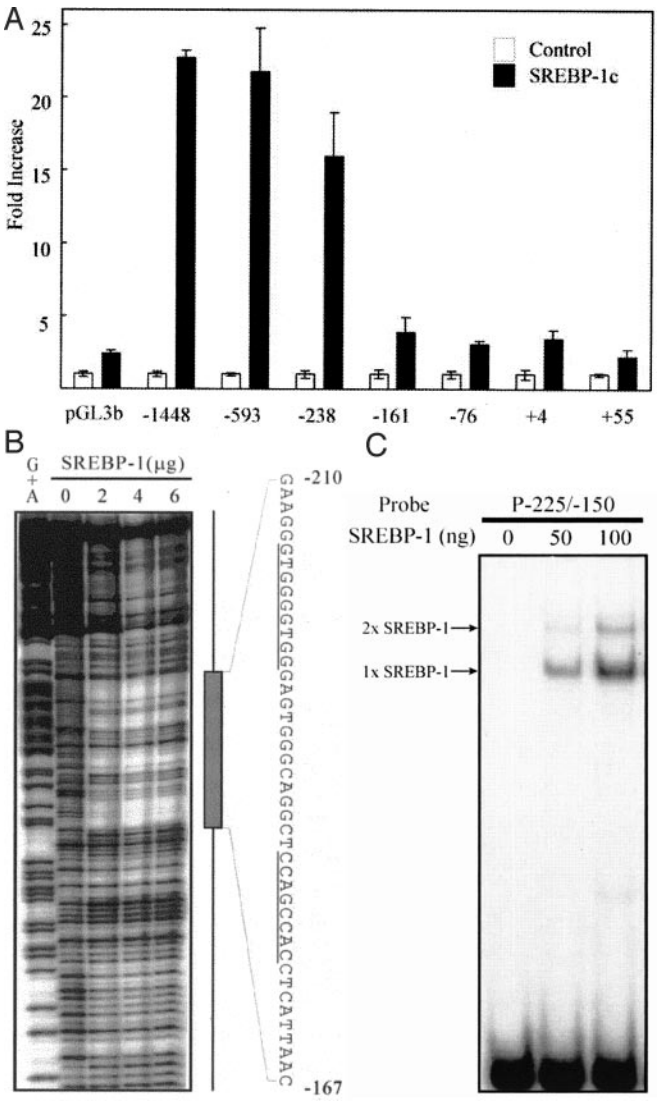

FIG. 2. **Localization of SRE in the LGK promoter.** *A*, 5′ serial deletion constructs of the LGK promoter luciferase reporter were transfected into Alexander cells with (*black bars*) or without (*white bars*) expression of SREBP-1c. 5′-ends of the promoters were shown. Normalized luciferase activities were expressed as fold increase relative to the basal activity in the absence of the SREBP-1c expression vectors and represented mean values ± S.D. from three independent experiments. *B*, DNase I footprinting assay was performed to localize SREBP-1 binding sites in the region between −238/-161 of the LGK gene. The DNA fragment from −320 to −69 of the LGK promoter was labeled by ^{32}P and mixed with recombinant SREBP-1 at protein concentrations (μg) noted above the respective lane. The *lane G+A* contains designated Maxam-Gilbert sequencing reaction products of the probe DNA. The regions protected from DNase I digestion were indicated by the *box*, and DNA sequences are shown. Two putative SREs are *underlined*. *C*, an EMSA using the −225/−150 region of the LGK promoter as a probe was performed. ^{32}P-labeled probe was incubated with recombinant SREBP-1 at the protein concentrations (ng) noted above the respective lanes. The shifted bands by SREBP-1 were indicated.

named pRGL-1448ma, pRGL-1448mb, and pRGL-1448mab, respectively. Mutations of SREa (pRGL-1448ma) resulted in 80% loss of SREBP-1c-dependent activation, and mutations of SREb (pRGL-1448mb) resulted in a 70% loss of activation. SREBP-1c could not activate the promoter-containing double mutations of SREa and SREb (pRGL-1448mab). These results indicated that the two functional SREs were closely related and necessary for the complete activation of the LGK promoter by SREBP-1c. To know whether the binding of SREBP-1 to one SRE could affect the binding to the other SRE, we performed EMSA using the probe covering the −225/−150 region of the LGK promoter. We

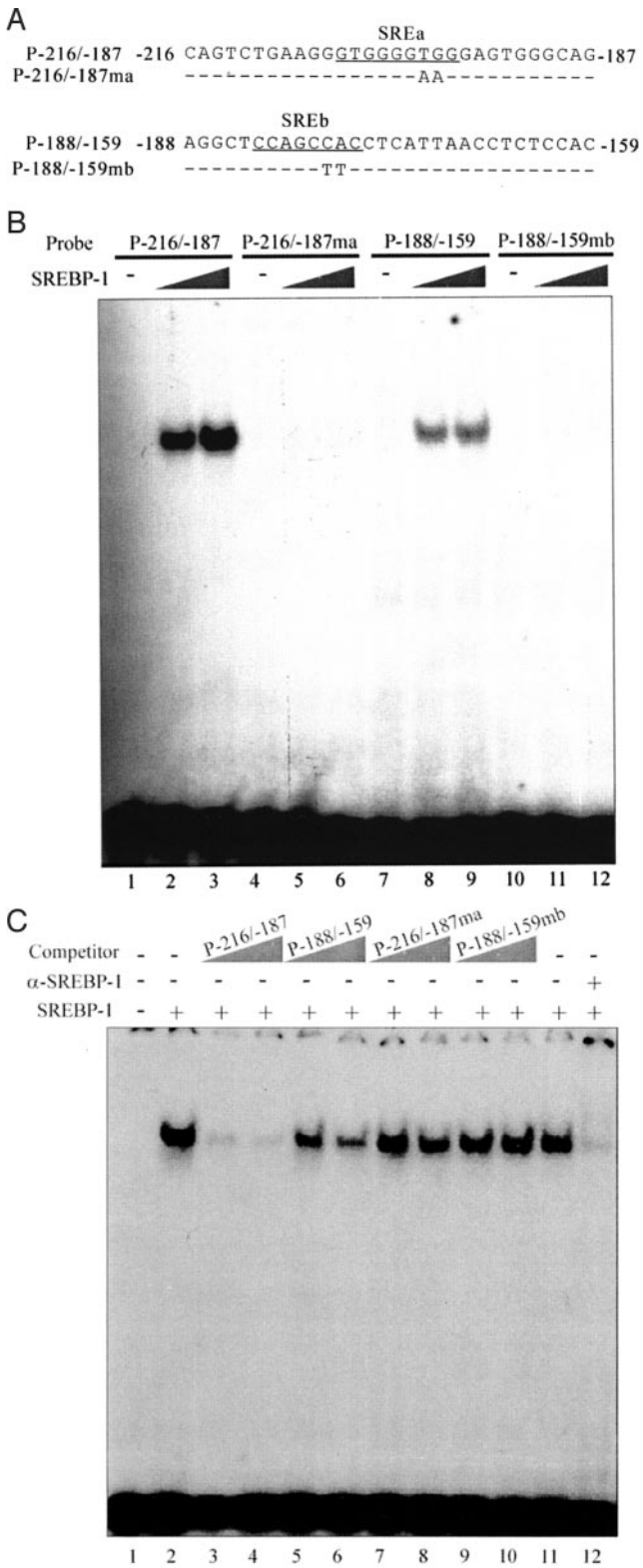

FIG. 3. **Identification of two distinct SREs in the LGK promoter.** *A*, DNA sequences of the wild type and mutant versions of the putative SREa and SREb are shown. *B*, EMSAs using the −216/−187 and −188/−159 regions of the LGK promoter and their mutant probes were performed. A ^{32}P-labeled probe was incubated with increasing amounts (50 and 100 ng) of recombinant SREBP-1 as indicated. Only one shifted band by SREBP-1 was shown. *C*, EMSA using P-216/-187 was performed to confirm the specificity of SREBP-1 binding. ^{32}P-labeled probe was incubated with 100 ng of recombinant SREBP-1 as noted above the respective lanes. Increasing amounts (50- and 100-molar excess) of unlabeled double strand oligonucleotides were added to the reaction mixture as indicated. An Anti-SREBP-1 serum was also added into reaction mixture as indicated.

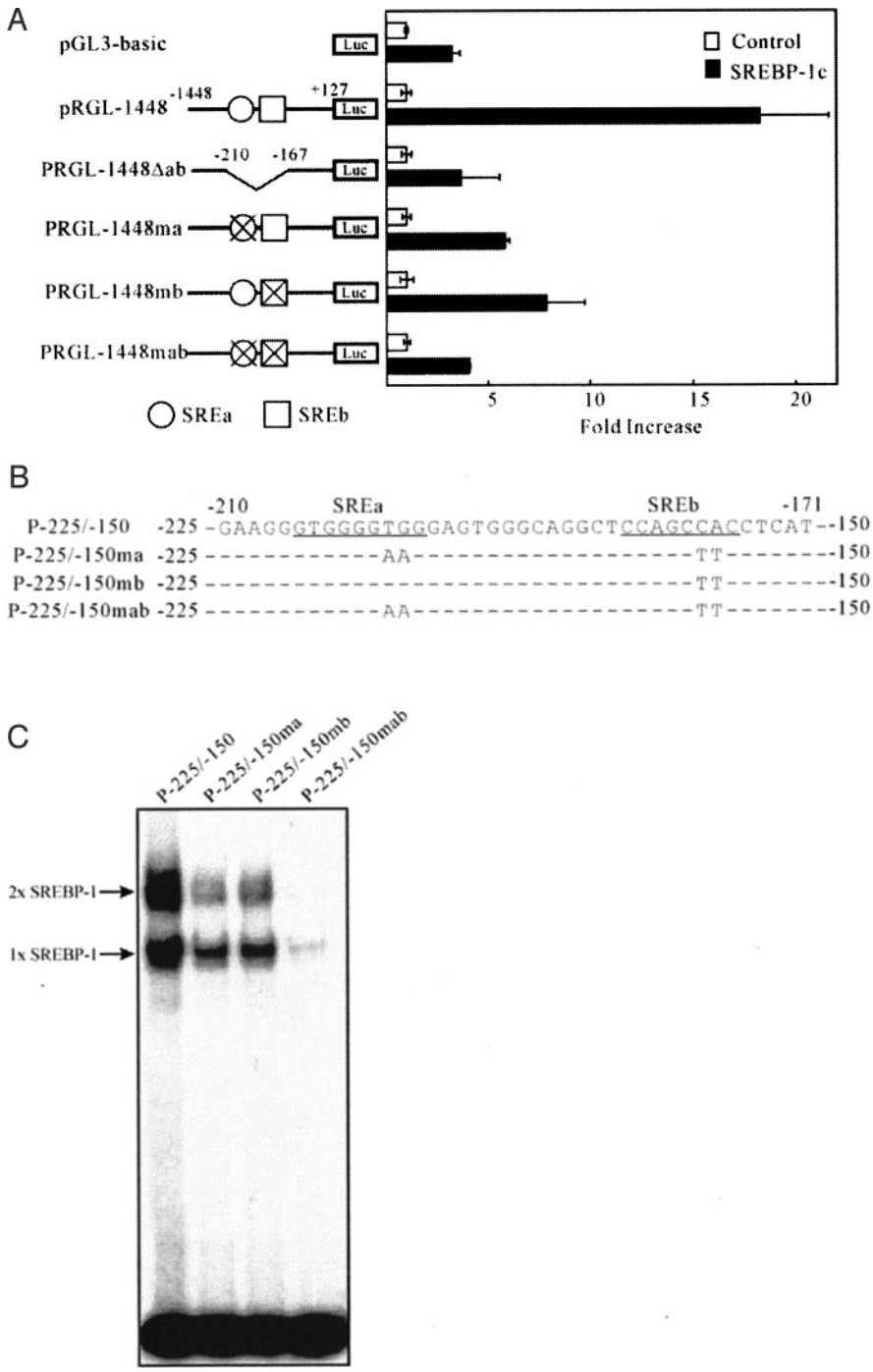

FIG. 4. **Functional characterization of SREs.** *A*, wild type or mutant LGK promoter luciferase reporter constructs were transfected into Alexander cells with (*black bars*) or without (*white bars*) overexpression of SREBP-1c. SREa and SREb are indicated by *circles* and *squares*, respectively, and mutations in SRE are indicated by ×. The truncated region in the promoter is also indicated. Normalized luciferase activities are expressed as the fold increase relative to the basal activity in the absence of the SREBP-1c expression vector and represent mean values ± S.D. from three independent experiments. *B*, structure of the P-225/-150 and its mutants of the putative SREa and SREb are shown. DNA sequences of −210/−171 is shown, and mutations are indicated by *underlining*. *C*, EMSA using P-225/-150 and its mutants were performed. ^{32}P-labeled probes were incubated with 100 ng of recombinant SREBP-1. The shifted bands by SREBP-1 are indicated.

used the P-225/-150, P-225/-150ma, P-225/-150mb, and P-225/-150mab as probes (Fig. 4*B*). Fig. 4*C* showed the change of the SREBP-1 binding pattern as a result of the mutations of SREs. The wild type probe, P-225/-150, showed two shifted bands of which the upper band was a DNA-protein complex containing two molecules of SREBP-1, whereas the lower band suggested one molecule of SREBP-1 binding. When SREa or SREb was mutated, the intensity of the lower band was moderately decreased, whereas the upper band almost disappeared. The binding of SREBP-1 to P-225/-150mb was stronger than that to P-225/-150ma. Double mutations in SREa and SREb resulted in a complete loss of SREBP-1 binding. These results suggested that SREBP-1 could bind to both SREa and SREb and that the bindings to these SREs were independent.

High Carbohydrate Diet Increased the Binding of SREBP-1c on LGK Promoter in Liver—In the liver the expression of LGK and SREBP-1c were known to be changed by nutritional status. Thus, we performed a ChIP assay to confirm the functionality of SREs *in vivo* and to determine whether the binding of SREBP-1c to the LGK promoter could be changed by nutritional status. As shown in Fig. 5*A*, a high carbohydrate diet dramatically increased the transcription of the GK and SREBP-1 in rat liver, but SREBP-2 transcription was not changed by nutritional status. In this state we performed a ChIP assay to find out whether a high carbohydrate diet could increase the binding of SREBP-1 on the LGK promoter. As shown in Fig. 5*B*, a high carbohydrate diet increased the binding of SREBP-1 to the −221/−169 region of the LGK promoter containing SREa and SREb. These results indicated that SREa and SREb were functional SREBP-1c binding sites *in vivo* and that a high carbohydrate diet increased SREBP-1 expression, thereby activating LGK expression in the liver.

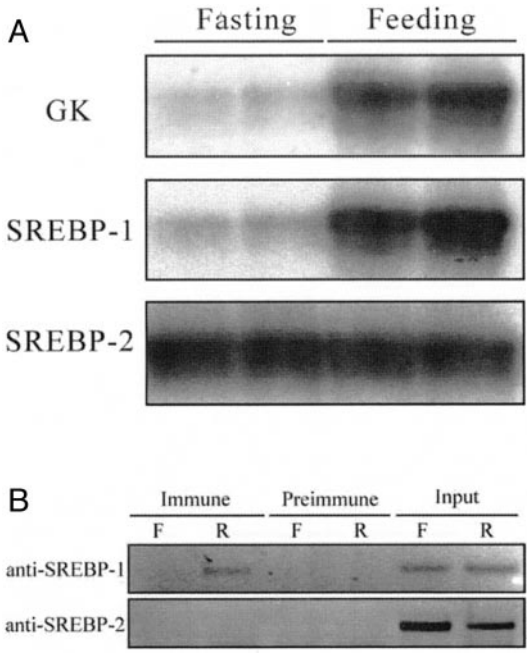

FIG. 5. **High carbohydrate diet increased the binding of SREBP-1c on LGK promoter.** Rats fasted for 24 h and were fed fat-free high carbohydrates for 24 h. Then the rats were sacrificed, and livers were removed at the 0- and 24-h time points. *A*, Northern blot analysis was performed to confirm the changes of the transcriptions of GK, SREBP-1, and SREBP-2. Total RNA (20 μg) isolated from the livers of each group was subjected to 1% formaldehyde agarose gel electrophoresis. RNA in the gel was transferred to a nylon membrane and hybridized to ^{32}P-labeled cDNAs for GK, SREBP-1, or SREBP-2. *B*, the association of SREBPs and the LGK promoter in rat liver was measured by ChIP assay. Livers of fasted (*F*) or refed (*R*) rats were cross-linked using formaldehyde. Chromatins of each group were incubated with anti-SREBP antiserum. DNAs in the presence or in the absence of antiserum were immunoprecipitated, and PCR amplification of the DNA fragments was conducted using primer pairs specific to the −320/−151 region of LGK. To confirm the same amounts of chromatins used in immunoprecipitation between groups, input chromatin was also used.

SREBP-1c Mediated the Transcriptional Activation of LGK by Insulin—It was reported that insulin regulates LGK gene expression and SREBP-1c mediates the action of insulin on LGK gene expression (23, 24). To determine whether insulin could activate the LGK promoter, we transfected LGK promoter luciferase reporter constructs into Alexander cells in the presence or absence of insulin. As shown in Fig. 6*A*, insulin increased the LGK promoter activity by 2.5-fold. However insulin did not activate the promoters lacking the SREs (pRGL-1448Δab and pRGL-161). These results suggested that SREa and SREb might be responsible for the insulin-dependent activation of the LGK promoter. Thus, we isolated primary hepatocytes from the liver of normal Sprague-Dawley rats and treated them with insulin to delineate the relationship between insulin, SREBP-1c, and GK. Fig. 6*B* shows the transcription of LGK and SREBP-1. Insulin selectively stimulated the transcription of LGK and SREBP-1c without changing the expression of SREBP-1a. A ChIP assay using the primary hepatocytes cultured in the presence or absence of insulin revealed that the binding of SREBP-1c to the −221/−169 region of the LGK promoter was increased by insulin (Fig. 6*C*). Despite these results, it is still possible that SREBP-1c may indirectly participate in the insulin-dependent activation of LGK. To find out whether SREBP-1c may directly mediate insulin-dependent LGK expression, we transduced an adenovirus containing SREBP-1c or SREBP-1cDN into the primary hepatocytes in the presence or absence of insulin. As shown in Fig. 6*D*, LGK expression is increased by adenoviral expression of SREBP-1c

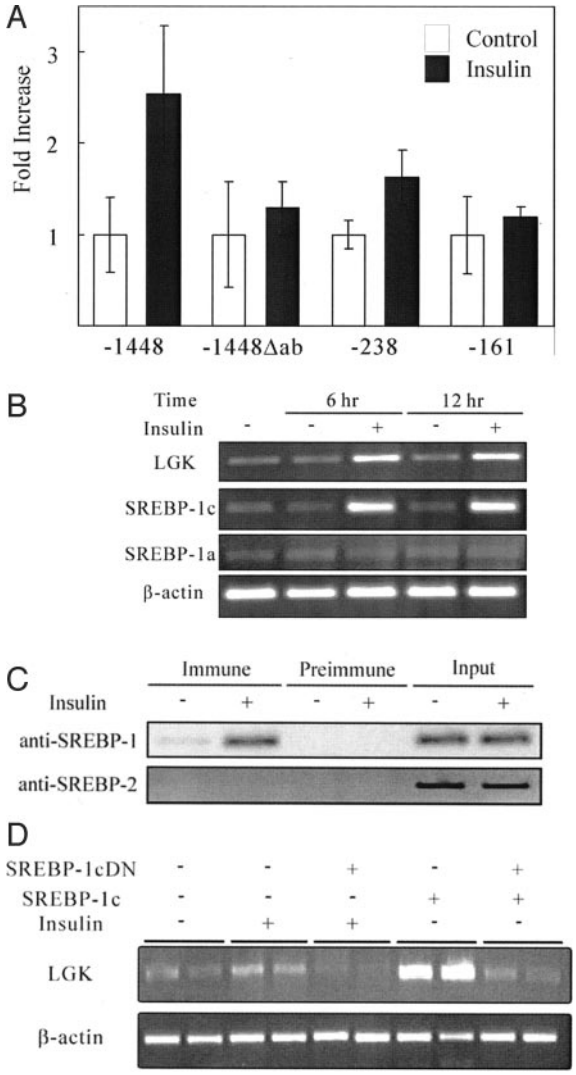

FIG. 6. **Insulin increased the binding of SREBP-1c on LGK promoter.** *A*, wild type or mutant LGK promoter luciferase reporter constructs were transfected into Alexander cells with (*black bars*) or without (*white bars*) insulin treatment (100 nM). *B*, primary hepatocytes isolated from rats were incubated in the presence or absence of insulin (100 nM). Semiquantitative analysis of LGK, SREBP-1a, and SREBP-1c was performed by reverse transcription PCR. *C*, a ChIP assay was adopted to measure the change of the binding of the SREBPs to SREs in response to insulin. Primary hepatocytes were incubated in the presence or absence of insulin for 12 h and cross-linked using formaldehyde. Chromatins of each group were incubated with anti-SREBP antiserum. DNA in the presence or absence of antiserum was immunoprecipitated, and PCR amplification of the DNA fragments was performed using primer pairs specific to the −320/−151 region of LGK. To confirm the same amounts of chromatins used in immunoprecipitation between groups, input chromatin was also used. *D*, adenovirus containing SREBP-1c or SREBP-1cDN or a null adenovirus was transduced into primary hepatocytes isolated from rats. The cells were then incubated in the presence or absence of insulin (100 nM) for 24 h. Semiquantitative analysis of LGK was performed by reverse transcription PCR. The quantity of the mRNAs was normalized with respect to β-actin mRNA.

in the absence of insulin, which is blocked by SREBP-1cDN. In addition, adenoviral expression of SREBP-1cDN could block the insulin-dependent stimulation of LGK gene expression. These results strongly suggested that SREa and SREb are responsible for the SREBP-1c-mediated activation of LGK gene expression by insulin.

DISCUSSION

Recently, SREBP-1c has been suggested to mediate the insulin action on hepatic GK transcription. SREBP-1c expression

is decreased in liver during fasting, which suppresses insulin and increases glucagon levels and is increased by refeeding (15, 25, 26). The expression of lipogenic genes are not increased by high-carbohydrate diet in SREBP-1 knock-out mice (27). SREBP-1c expression is very low in the liver of streptozotocin-induced diabetic rats and markedly increased by insulin (23). Adenoviral expression of SREBP-1c in the liver of streptozotocin-induced diabetic mice increased lipogenic gene expression, mimicking the effects of insulin (28). Overexpression of the dominant negative mutant of SREBP-1c blocked the insulin-dependent activation of GK and lipogenic gene expression in the liver (24, 28). In isolated rat hepatocytes, insulin treatment increased the amount of mRNA for SREBP-1c in parallel with the mRNAs of lipogenic genes, and glucagons can antagonize insulin action (23, 24, 26). Induction of lipogenic genes can be blocked if a dominant negative form of SREBP-1c is expressed (26). In addition, SRE has been identified in many promoters of lipogenic genes such as fatty acid synthase, acetyl-CoA carboxylase, ATP citrate-lyase, and S14, and insulin-responsive regions of fatty acid synthase and S14 genes are colocalized with functional SREBP-1c binding sites (17, 29–33). However, neither the insulin-responsive region nor the SREBP response element has been identified in the LGK promoter.

In the present study, we identified two functional SREBP-1c response elements, SREa and SREb, in the rat LGK promoter. SREBP-1c binds to these SREs and activates the LGK promoter. In an EMSA, the binding of SREBP-1 to SREa or SREb was not mutually exclusive. SREa showed a stronger affinity to SREBP-1 compared with SREb. However, these *in vitro* binding assay data were not in parallel with the transfection assay. Transient transfection assay showed that both SREa and SREb were necessary for the transactivation of LGK promoter by SREBP-1c, and the physical interaction between the protein and SREs of the LGK promoter *in vivo* was confirmed by ChIP assay. A high carbohydrate diet increased the expression of SREBP-1 and GK in rat liver and the binding of SREBP-1 to the LGK promoter. Given that SREBP-1c was a major form of SREBP-1 and that the expression of SREBP-1c was known to be regulated by insulin in liver, the increased GK expression by high carbohydrate feeding might be due to the increased binding of SREBP-1c to the LGK promoter. We also showed evidence supporting this hypothesis using primary hepatocytes. Insulin selectively increased SREBP-1c and LGK expression in primary cultured hepatocytes, which was blocked by adenoviral expression of the dominant negative mutant of SREBP-1c. A ChIP assay using primary hepatocytes showed the increased binding of SREBP-1 to SREs of LGK promoter. From these results, we could conclude that the binding of SREBP-1c to SREa and SREb is responsible for insulin-dependent LGK expression.

In this study, it was not possible to explain the detailed relationship between SREa and SREb in SREBP-1c-mediated activation of LGK by insulin. There may be some unknown or additional mediators that play important roles in SRE-mediated transactivation of the LGK gene. We thought that there might be something more than SREBP-1c linking SREa and SREb in the regulation of LGK promoter by SREBP-1c. When pRGL-1448 was transfected into Alexander cells or primary hepatocytes, the promoter showed only 2.5-fold activation by insulin, which is much smaller than the activation of LGK promoter by SREBP-1c. This marginal activation suggested that other regions outside the −1448/+127 region or other factors that are lacking in these experimental conditions might involve the increase of SREBP-1c binding to SREa and SREb by insulin *in vivo*.

In conclusion, we identified functional SREs in the rat LGK promoter and demonstrated that SREBP-1c is the transcription factor mediating insulin action on LGK expression. Although the mutual interaction of these two *cis*-elements in activating LGK gene expression remains elusive, the tandem array of SREa and SREb may provide a fine control for regulating glucose homeostasis by LGK.

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Additions and Corrections

Vol. 278 (2003) 50702–50713

Temporal and spatial modulation of Rho GTPases during *in vitro* formation of capillary vascular network. Adherens junctions and myosin light chain as targets of Rac1 and RhoA.

Ilaria Cascone, Enrico Giraudo, Francesca Caccavari, Lucia Napione, Elisa Bertotti, John G. Collard, Guido Serini, and Federico Bussolino

Page 50711: Fig. 9*D* was inadvertently omitted. The figure and legend are shown on facing page.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

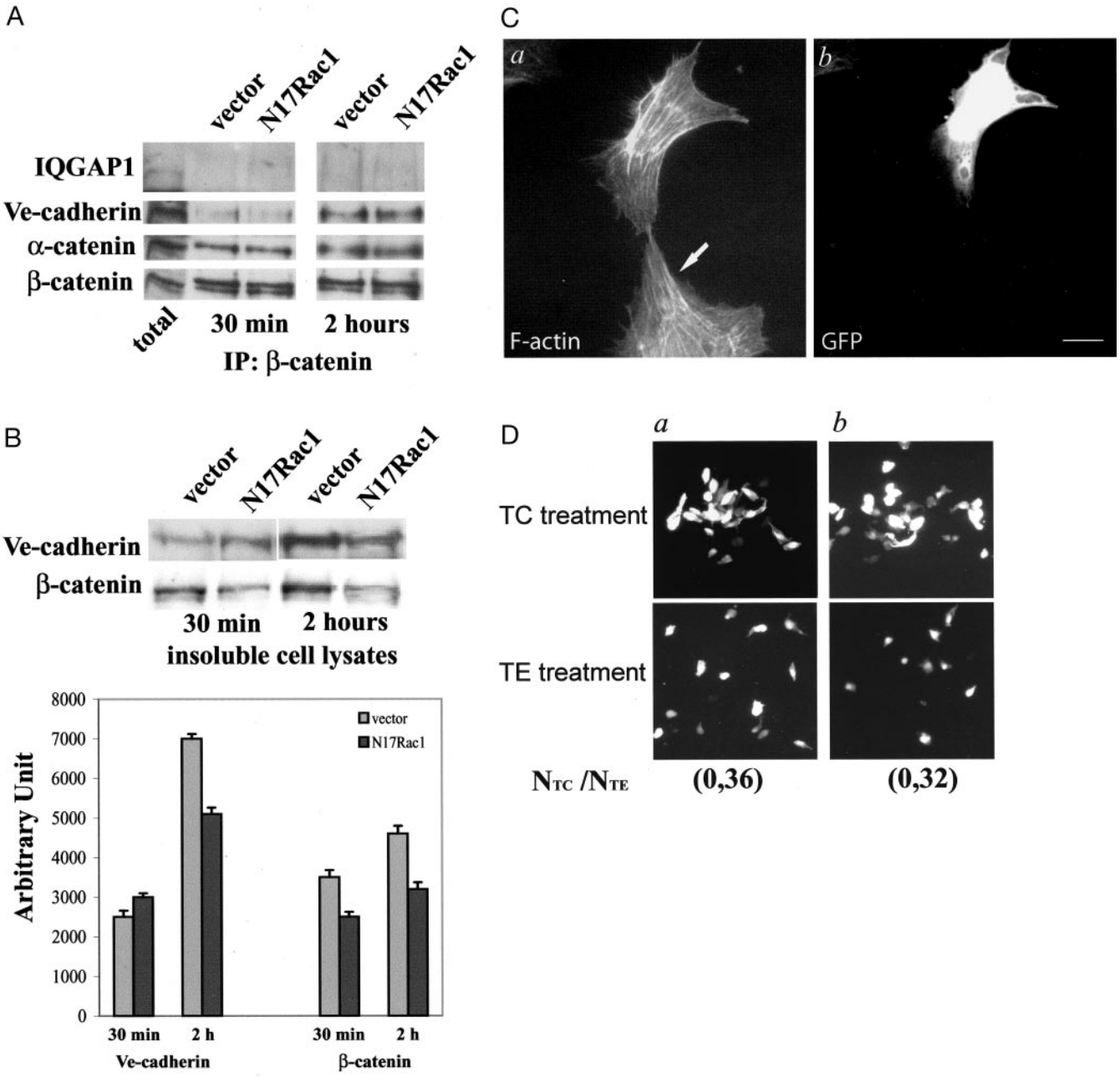

FIG. 9. **Effect of the expression of N17Rac1 on the dynamic of cell-to-cell adherens junctions.** *A*, capillary ECs carrying vector alone or N17Rac1 were plated on Matrigel and dispersed with MatriSperse cell recovery solution at 30 min or at 2 h from the beginning of the morphogenetic process, lysed, and immunoprecipitated (*IP*) with mAb anti-β-catenin. Proteins were separated by SDS-PAGE (8%) and then immunoblotted with Abs anti-IQGAP-1 and anti-α-catenin and mAbs anti-Ve-cadherin and anti-β-catenin. The total amounts in vector-ECs of the indicated proteins are shown in the *1st lane*. Equal results have been obtained in N17Rac1-ECs (not shown). *B*, ECs carrying vector alone or N17Rac1 were recovered as indicated above and lysed. After centrifugation, Triton X-100-insoluble pellets were solubilized in SDS buffer detailed under "Experimental Procedures" and boiled for 5 min. Solubilized proteins were separated on SDS-PAGE 8%) and immunoblotted with mAbs anti-Ve-cadherin or anti-β-catenin. Densitometric analysis of three independent experiments is shown as mean ± S.D. *C*, ECs carrying N17Rac1 were plated on Matrigel and after 2 h fixed and stained with TRICT-phalloidin (*a*). *b* shows the GFP expression in one of the two cells recorded in *a*. The negative GFP cell, which does not express N17Rac1, shows actin cable near and perpendicular to the cell protrusion forming the contact with a GFP-, N17Rac1-expressing cell. In this cell, F-actin is absent at the protrusion cone. Four experiments with similar results were performed. *D*, confluent cultured ECs carrying vector alone (a) or N17Rac1 (*b*) were treated with 0.01% trypsin in Hepes-buffered (TC treatment) or in Hepes-buffered saline without Ca^{2+} supplemented with 1 mM EGTA (TE treatment) for 15 min at 37 °C and dissociated through 10 times pipetting. The extent of dissociation cells was represented by the index N_{TC}/N_{TE}, where N_{TC} and N_{TE} are the number of clusters and single cells, respectively. This figure is representative of three experiments obtained with similar results.

Vol. 279 (2004) 29031–29042

Single nucleotide polymorphism (−468 Gly to Ala) at the promoter region of sterol regulatory element-binding protein-1c associates with genetic defect of fructose-induced hepatic lipogenesis.

Ryoko Nagata, Yoshihiko Nishio, Osamu Sekine, Yoshio Nagai, Yasuhiro Maeno, Satoshi Ugi, Hiroshi Maegawa, and Atsunori Kashiwagi

The title was incorrect. The correct title should be:

Single nucleotide polymorphism (−468 G to A) at the promoter region of SREBP-1c associates with genetic defect of fructose-induced hepatic lipogenesis

Vol. 279 (2004) 30823–30829

SREBP-1c mediates the insulin-dependent hepatic glucokinase expression.

So-Youn Kim, Ha-il Kim, Tae-Hyun Kim, Seung-Soon Im, Sang-Kyu Park, In-Kyu Lee, Kyung-Sup Kim, and Yong-Ho Ahn

Page 30827, Fig. 4*B*: In Fig. 4*B*, the sequence of oligonucleotide P-225/-150 is shown in the *top line*, and SREa and SREb are marked by *underlines*. The *lower three lines* are mutated oligonucleotides. There is a mistake in the second line, which indicates the oligonucleotide-containing mutation of SREa. The sequence of P-225/-150ma is equal to P-225/-150mab. "TT" of the SREb position should be changed by – –. The corrected figure is shown below.

FIG. 4