Sterol Regulatory Element–Binding Protein-1 Mediates the Effect of Insulin on Hexokinase II Gene Expression in Human Muscle Cells

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Insulin upregulates hexokinase II (HKII) expression in skeletal muscle, and this effect is altered in type 2 diabetic patients. This study was conducted to identify the transcription factors that mediate the effect of insulin on HKII gene expression in human muscle. We have cloned the promoter region of the HKII gene and investigated its regulation in a primary culture of human skeletal muscle cells. We defined a region (−369/−270) that conferred the transcriptional response to insulin. This region contains a sterol regulatory element (SRE) that interacted with the recombinant active form of SRE binding protein-1c (SREBP-1c) in electrophoretic mobility shift assays, and, using chromatin immunoprecipitation assay, we showed that endogenous SREBP-1 interacted directly with the promoter region of the HKII gene in human muscle cells. Mutation of the SRE sequence completely suppressed the response of the promoter to insulin stimulation. Finally, overexpression of the rodent mature form of SREBP-1c (adipocyte determination and differentiation factor-1 [ADD1]-403) was able to reproduce insulin action, whereas a dominant-negative form (ADD1-403R) prevented the effect of insulin on HKII promoter constructs. These results demonstrate that SREBP-1c is involved in the effect of insulin on HKII gene transcription and indicate that it is one of the mediators of insulin action on gene expression in human skeletal muscle. *Diabetes* 53:321–329, 2004

The different hexokinases (ATP:D-hexose-6-phosphotransferase EC 2.7.1.1) convert glucose into glucose-6-phosphate and thus catalyze the first rate-limiting step of glucose metabolism in the cells (1). Hexokinase II (HKII) is the predominant isoform expressed in skeletal muscle, heart, and adipose tissue (1). In these tissues, glucose uptake is strongly activated by insulin, which induces the translocation of the GLUT4 transporter to the plasma membrane. Simultaneously, insulin increases HKII catalytic activity (2,3), allowing an immediate metabolism of glucose in the cells. In addition, insulin increases the expression of HKII mRNA and protein, as has been demonstrated in rat (3,4) and human tissues (5–7). Interestingly, the expression and activity of HKI, the other isoform expressed in muscle and adipose tissue, is not affected by insulin, indicating a specific action of the hormone on the HKII gene (2,4,6).

Type 2 diabetes is characterized by impaired insulin-stimulated glucose utilization (8), and studies using nuclear magnetic resonance have found evidence of a defect in glucose transport and/or phosphorylation steps in the skeletal muscle of the patients (9). Although GLUT4 expression is not altered, a marked reduction in HKII steady-state mRNA level and HKII activity have been consistently reported in the muscle of diabetic patients (5–7). Furthermore, the regulation of HKII expression by insulin was found to be altered in the skeletal muscle of type 2 diabetic subjects (6,7). These data prompted us to better define the mechanism by which insulin regulates the expression of HKII in human muscle.

Using rat L6 myotubes, it was recently demonstrated that the phosphatidylinositol (PI) 3-kinase/p70-S6 kinase pathway is required for the regulation of HKII gene transcription by insulin (10). However, the transcription factors mediating the insulin signal at the promoter level have not yet been identified. Insulin has a major effect on gene expression in skeletal muscle (11), but no consensus insulin response elements have been found in the regulatory sequences of these genes, and the involved transcription factors have not been fully characterized (11).

To determine the mechanism of insulin action on gene expression in skeletal muscle, we attempted, in the present study, to identify the promoter region of the human HKII gene and the transcription factors involved in the effect of insulin using a primary culture of human muscle cells. A number of studies have demonstrated the usefulness of this cell model to investigate the action of insulin on glucose metabolism and on the regulation of gene expression (12–15), and it has been consistently reported that differentiated myotubes display numerous features of mature skeletal muscle (12,16). Using different constructs of the 5′-flanking region of the human HKII gene, we identified a region of the promoter that confers insulin response and demonstrated that sterol regulatory element (SRE) binding protein-1 (SREBP-1) is involved in the effect of insulin in human muscle cells.

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ADD1, adipocyte determination and differentiation factor-1; ChIP, chromatin immunoprecipitation; FAS, fatty acid synthase; HK, hexokinase; PI, phosphatidylinositol; SRE, sterol regulatory element; SREBP, SRE binding protein.

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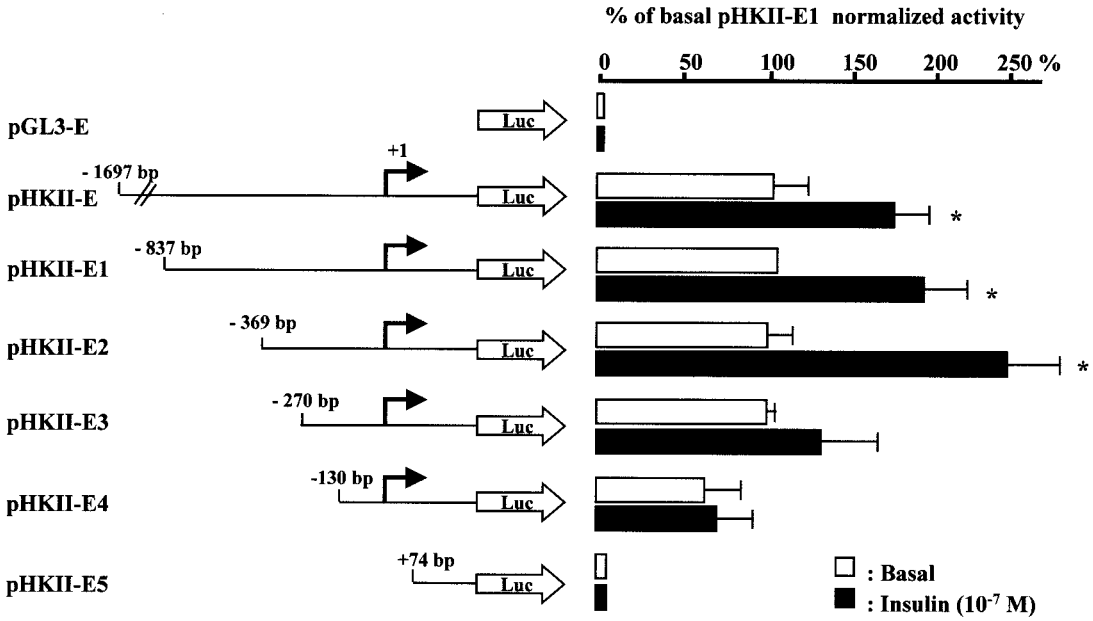

FIG. 1. Insulin transactivates the promoter of the HKII gene in human muscle cells. Differentiated human skeletal muscle cells were transfected with a luciferase reporter gene driven by different construction of the human HKII promoter. Serum-starved cells were incubated for 24 h with or without insulin (10^{-7} mol/l). Relative luciferase activity was calculated using a dual luciferase assay as indicated in RESEARCH DESIGN AND METHODS. Data are expressed in terms of the basal luciferase activity of pHKII-E1 and are presented as the means ± SE for at least five different transfection experiments for each construct. *$P < 0.05$ in the presence of insulin versus in the absence of insulin using a paired t test.

RESEARCH DESIGN AND METHODS

Culture media and products. Culture media were from Invitrogen (Cergy Pontoise, France). FCS was purchased from Biomedia (Boussens, France). Porcine insulin was purchased from Sigma (La Verpillere, France).

Primary culture of human skeletal muscle cells. Biopsies of the erector spinae skeletal muscle (~1 g) were taken, with the consent of the subjects, during surgical procedure. In the present study, muscle samples were taken from healthy lean subjects (49 ± 5 years) with no familial or personal history of diabetes, dyslipidemia, or hypertension. The Ethics Committee of Lyon Hospitals approved the experimental protocol. The satellite cells were isolated by trypsin digestion and were grown as described previously in detail (12,13). Confluent myoblasts were allowed to differentiate into myotubes for 7 days (12,16). The differentiated cells expressed muscle-specific markers and were characterized by insulin-sensitive glucose transport (12,16). The human myotubes were maintained in serum-free medium for 24 h before treatment with insulin.

Plasmid constructions and site-directed mutagenesis. A cDNA fragment of the HKII promoter (−2,551/+103) was obtained by PCR using human genomic DNA and subcloned into pGEM-T plasmid (Promega, Charbonniere, France). A fragment of the 5′ untranslated region (+74/+439) was also obtained by PCR and cloned into the pBluescript KS+ plasmid (Stratagene, La Jolla, CA). Their sequences (ALFexpress AutoRead sequencing kit; Amersham Pharmacia Biotech, Upsala, Sweden) perfectly matched the sequences accessible in GenBank (AC034288 clone RP11–249E10, AF148513) and the published data (17). A −1,697/+439 fragment was generated by digestion/ligation and subcloned into the promoterless luciferase reporter gene vector pGL3-Enhancer (Promega) to obtain pHKII-E. The pHKII-E1 construct (−837/+439) was then generated from pHKII-E vector by restriction enzyme digestion (*Bcl*I). Because of the lack of useful restriction sites, pHKII-E2 (−369/+433), pHKII-E3 (−270/+433), and pHKII-E4 (−130/+433) were generated by PCR. PHKII-E5 was obtained from pHKII-E4 by deleting the −137/+74 region (*Apa*I digestion). Finally, the pHKII-E2m construct was generated by site-directed mutagenesis from pHKII-E2 (QuickChange site-directed mutagenesis kit; Stratagene). The sequence of the mutated oligonucleotide (msre1) is indicated in Fig. 2. The sequence of each construct was verified before being used.

Measurement of HKII promoter activity. Myotubes were maintained in serum-free medium for 18–24 h before transfection using ExGen 500 reagent (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. Each culture well received 500 ng of a given HKII promoter/luciferase gene construct mixed with 2 ng of pRL-CMV vector (Promega). At 6 h after transfection, the medium was changed (α-minimal essential medium without FCS and 1% antibiotics), and cells were incubated with or without insulin (10^{-7} mol/l) for 24 h. Firefly and *Renilla* luciferase activities (dual luciferase reporter assay system; Promega) were then measured using a TD-20/20 luminometer (Turner Designs) without delay and during a 15-s measuring period. All of the transfection experiments were performed in duplicate and repeated at least three times in independent experiments.

Overexpression of ADD1/SREBP-1c in human skeletal muscle cells. For SREBP-1c overexpression, 500 ng of pSVSport1-adipocyte determination and differentiation factor-1 (ADD1) (403) or pSVSport1-ADD1(403R) (gift of Dr. B. Spiegelman, Harvard Medical School, Boston, MA) was cotransfected with the promoter construct plasmids before transfection. The amount of DNA was kept constant by adding pBluescript KS+ plasmid when necessary.

Preparation of human SREBP-1c expression vector. A partial cDNA encoding SREBP-1c was obtained by RT-PCR from human muscle total RNA, using 5′-GCAGATCGCGGAGCCATGGATTGC-3′ as sense primer and 5′-CTA CACTGCCACAGGCCGACACC-3′ as antisense primer, and then cloned in the pCDNA-3 expression vector (Stratagene). The antisense primer was modified to generate a stop codon just after the codon corresponding to amino acid 382. The resulting cDNA (pCDNA-h382SREBP1c) codes approximately for the nuclear form (after cleavage) of the native human SREBP-1c protein (accession no. U00968).

Electrophoretic mobility shift assay. Double-stranded oligonucleotides corresponding to the sequences of putative SRE binding sites (sre1, sre2, msre1, FAS-SRE, and FAS-mSRE) (sequences indicated in Fig. 2), were labeled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (ICN Pharmaceuticals, Orsay, France). ADD1-403, ADD1-403R, and h382SREBP-1c proteins were produced by in vitro translation using the TNT T7/SP6-coupled reticulocyte lysate system (Promega). Nuclear protein extracts from myotubes were prepared according to Azzout-Marnich et al. (18). For binding assays, reactions were carried out for 30 min at 4°C in 20 μl containing 20% glycerol, 2 mmol/l dithiothreitol, 40 mmol/l HEPES (pH 7.9), 10 mmol/l $MgCl_2$, 0.4 mmol/l EDTA, 400 mmol/l KCl, and 1 μg of poly(dIdC) and supplemented with either the in vitro–translated protein lysate (1–8 μl) or the nuclear protein extract (15 μg of protein) and ~80,000 cpm of 5′-end ^{32}P-labeled oligonucleotide probes. DNA-protein complexes were resolved in a nondenaturing 5% PAGE and visualized using an SI phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed according to Orlando et al. (19). Briefly, formaldehyde cross-linked chromatin extracts were prepared from human myotubes and fragmented by sonication. First, 20 μg of chromatin extract were precleared with protein A Sepharose beads (Amersham Biosciences, Orsay, France) for 1 h. After centrifugation, supernatants were incubated overnight at 4°C with 10 μg of an anti–SREBP-1 antibody (reference H-160; Santa Cruz Biotechnology, Santa Cruz, CA), an anti-RNA polymerase II antibody (reference 8WG16; Convance, Berkeley, CA) or no antibody (mock condition). The immunoprecipitated DNA/protein complex were bound to

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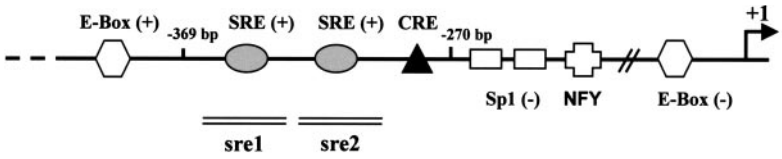

Name	5'-3' sequence	position
SRE	ATCACNCCAC	M00221
sre1	ttttcca-GTCGCCCCAC-accccgg	-346/-323
sre2	gatcacg-CTCCCCCCAC-ccatagc	-316/-293
msre1	ttttcca-ATTATCTCAC-accccgg	-351/-318
FAS-SRE	gcgcggg-ATCACCCCAC-cgacggc	[see Ref (22)]
FAS-mSRE	gcgcggg-ATTATCTCAC-cgacggc	[see Ref (22)]

FIG. 2. Schematic representation of the potential transcription factor binding sites located in the −369 to −270 region. The location of the SREBP, Sp1, NFY, CRE (cAMP response element), and E-box elements are indicated in the upper part of the figure. The table shows the consensus sequence of the SRE and the sequences of the different oligonucleotides used in the present work. The nucleotides of the SRE consensus sequence are indicated in bold, with conserved nucleotides in capitals. FAS-SRE, SRE motif identified in the rodent FAS promoter (22).

protein A Sepharose beads after 3 h of incubation at 4°C and washed in a low-salt buffer, high-salt buffer, LiCl buffer, and Tris-EDTA buffer in succession as described by Duong et al. (20). Proteins were eliminated using proteinase K (200 μg; Promega) in the presence of 10% SDS by overnight incubation at 37°C. After phenol extraction, the DNA was precipitated, suspended in water, and used as a template for PCR. The sets of PCR primers used for the analysis of the HKII proximal promoter were 5′-GGCCGTGCTA CAATAGGTAAGAAC-3′ (p1) (Fig. 5), 5′-GGACGGAAAGGCGCA TGAGC CAC-3′ (p2) or 5′-GAGCCGTGAGCGATGATTGGC-3′ (p3), 5′-GAGTTTC ATGCAACAATGTGGCTT-3′ (p4). Primers used for the analysis of the exon 2 of the HKII gene (negative control) were 5′-GTTGACCAGTATCTCTACCAC-3′ (p5) and 5′-CTGTCCCATCTGGAGTGGACCTC-3′ (p6). PCR products were analyzed on ethidium bromide–stained 3% agarose gels.

SREBP-1c protein quantitation in human skeletal muscle cells. Membrane fractions and nuclear protein extracts from unstimulated and insulin-treated myotubes were prepared according to Azzout-Marnich et al. (18). Proteins from nuclear extracts and membrane fractions were resolved in 10% SDS–polyacrylamide gel. SREBP-1 proteins (precursor and active forms) were detected using a mouse monoclonal antibody (IgG-2A4) raised against amino acids 301–407 of human SREBP-1 (American *Type Culture* Collection, Manassas, VA) and visualized using an enzyme-catalyzed fluorescence substrate for Western blotting (Amersham Pharmacia Biotech).

RESULTS

Functional characterization of the human HKII promoter region. Primary cultures of differentiated human skeletal muscle cells have been previously shown to be suitable for the study of insulin action on both glucose metabolism (16) and gene expression (12,13). We previously reported that HKII mRNA is expressed at similar levels in myotubes and in human skeletal muscle and that insulin induced its expression in a time-dependent manner (approximately twofold increase in HKII mRNA level after 6 h of stimulation) (21). To identify the regions of the human HKII gene promoter involved in the effect of insulin, luciferase expression vector, driven by various fragments of the 5′-flanking region, were transfected into human myotubes. In the basal state, cells transfected with the pHKII-E1 construct (−837/+433) showed a ~50-fold increase in luciferase activity when compared with transfection with the promoterless pGL3-Enhancer vector (50.2 ± 5.7, $n = 9$ experiments, data not shown). For the presentation of the results, the luciferase activity of the other constructs, measured in the absence or presence of insulin, were expressed in reference to pHKII-E1. In the absence of insulin, Fig. 1 shows that the longer construct (pHKII-E) has activity similar to that of pHKII-E1. The progressive deletions of the 5′ end of the HKII promoter did not affect luciferase activity until the deletion of the sequence between −270 and −130 from the transcription initiation site. This deletion was associated with an approximately twofold reduction in luciferase activity. As expected, the removal of the transcription initiation site (pHKII-E5) completely suppressed the promoter activity.

Insulin treatment (10^{-7} mol/l) significantly increased (1.6 ± 0.2-fold, $P = 0.001$) luciferase activity in myotubes transfected with the pHKII-E construct (Fig. 1). The stimulation of promoter activity by insulin was also observed with pHKII-E1 (1.87 ± 0.26-fold, $P < 0.001$) and pHKII-E2 (2.12 ± 0.43-fold, $P = 0.007$). Further deletion of the 5′ region (pHKII-E3) abolished the effect of insulin on HKII promoter activity (Fig. 1). This result indicated that the region spanning −369 to −270 contains *cis*-acting sequences responsible for the response of the HKII promoter to insulin in human skeletal muscle cells.

To verify whether the 5′-untranslated region could also play a role in the effect of insulin, the +74/+433 region was deleted in pHKII-E1. This resulted in a marked reduction in luciferase activity (~60%), but the stimulatory effect of insulin was still observed (1.55 ± 0.13, $P = 0.001$), suggesting that the 5′-untranslated region is not involved in insulin action on the HKII promoter (data not shown).

Analysis of the −369 to −270 region of the HKII promoter. The sequence of the region conferring insulin response was analyzed using TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) and Genomatix (www.genomatix.de). As indicated in Fig. 2, in this region we identified two potential binding sites for SREBP-1 (SRE), a putative recognition site for cAMP response element, an E-box motif (−410/−404), as well as two Sp1 motifs (−255/−247 and −216/−207) and an NFY motif (−126/−113). The two potential SREBP-1 binding sites (SRE) on the HKII promoter were located at −339/−330 and −309/−300. When compared with the SRE's core sequence found in the promoter of fatty acid synthase (FAS), a gene known to be regulated by SREBP-1c in response to insulin (22), there was a G instead of a A in positions 1 and 4 for the −339/−330 SRE and a C instead of a A in positions 1 and 4 for the −309/−300 SRE (Fig. 2).

We therefore studied in more detail the possible impli-

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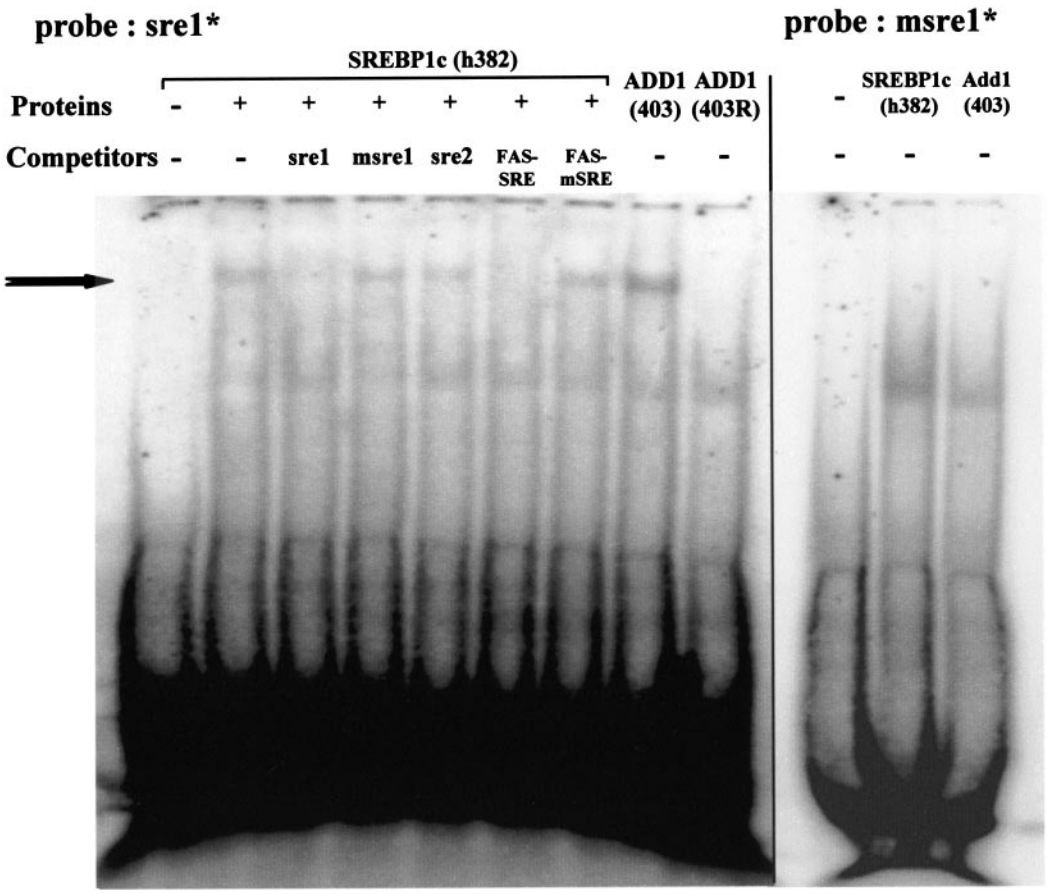

FIG. 3. Electrophoretic mobility shift analysis demonstrating binding of SREBP-1c recombinant proteins to the −339/−330 SRE of the HKII promoter. Electrophoretic mobility shift assays were performed with 5′ end–labeled sre1 oligonucleotide in the presence of in vitro–synthesized h382SREBP-1c, ADD1-403, or ADD1-403R proteins as described in RESEARCH DESIGN AND METHODS. Competition was performed with 100-fold excess of the indicated unlabeled oligonucleotides (sequences in Fig. 2). The arrow indicates the specific complex. *Probes radioactively labelled with ^{32}P.

cation of these two putative SREs in the effect of insulin on HKII promoter activity. We first verified whether they could bind SREBP-1 proteins using electrophoretic mobility shift assay. Two probes, sre1 (−346/−323) for the −339/−330 SRE and sre2 (−316/−293) for the −309/−300 SRE (sequences in Fig. 2), were designed, labeled, and studied for their ability to interact with in vitro–translated proteins corresponding to either h382SREBP-1c, ADD1–403, or ADD1-403R. Figure 3 shows the results obtained with the sre1 probe. Both h382SREBP-1c and ADD1-403 formed complexes with sre1 (*lanes 2* and *8*), whereas ADD1-403R did not bind the probe (*lane 9*). There was competition for the interaction with h382SREBP-1c by an excess of unlabeled sre1 probe (*lane 3*), but not by msre1, which corresponded to a mutated sre1 probe (*lane 4*). As shown in the right part of Fig. 3, msre1 was not able to interact with recombinant h382SREBP-1c or ADD1-403 proteins. We also used a probe (FAS-SRE) designed to bind SREBP-1 protein in the FAS promoter (22). We found that this probe competed with sre1 for the binding of h382SREBP-1c (*lane 6*). When this probe was modified (FAS-mSRE) to reproduce the mutations that were previously shown to abolish the interaction of SREBP-1 with the FAS promoter (22), there was no competition with sre1 for the binding of h382SREBP-1c (*lane 7*). It could be added that FAS-SRE, but not FAS-mSRE, interacted with both h382SREBP-1c and ADD1-403 proteins, and there was competition for these interactions by the sre1 probe (data not shown).

In contrast to the sre1 oligonucleotide, the sre2 probe did not form a specific complex with either h382SREBP-1c or ADD1-403 (data not shown). Moreover, excess sre2 probe did not compete with sre1 for the binding of h382SREBP-1c (Fig. 3, *lane 5*). These results indicated, therefore, that despite of strong homology between the SRE elements of sre1 and sre2, only sre1 is able to bind SREBP-1 recombinant proteins, suggesting that only the SRE at position −339/−330 could be functional in the human HKII promoter.

We next verified whether proteins in nuclear extract from human skeletal muscle cells were also able to bind the same region of the HKII promoter. As shown in Fig. 4, electrophoretic mobility shift assay using the sre1 probe resulted in the formation of a complex in the presence of nuclear extract from human myotubes. The size of the complex was similar to the one formed with recombinant h382SREBP-1c, and an excess of unlabeled probe abolished the interaction, whereas the mutated probe (msre1) did not compete with sre1 (Fig. 4). These data suggested that SREBP-1 from nuclear extract binds the sre1 sequence. However, because we were not able to obtain supershift using either the monoclonal (IgG-2A4) or the polyclonal (H-160) anti–SREBP-1 antibody, this hypothesis

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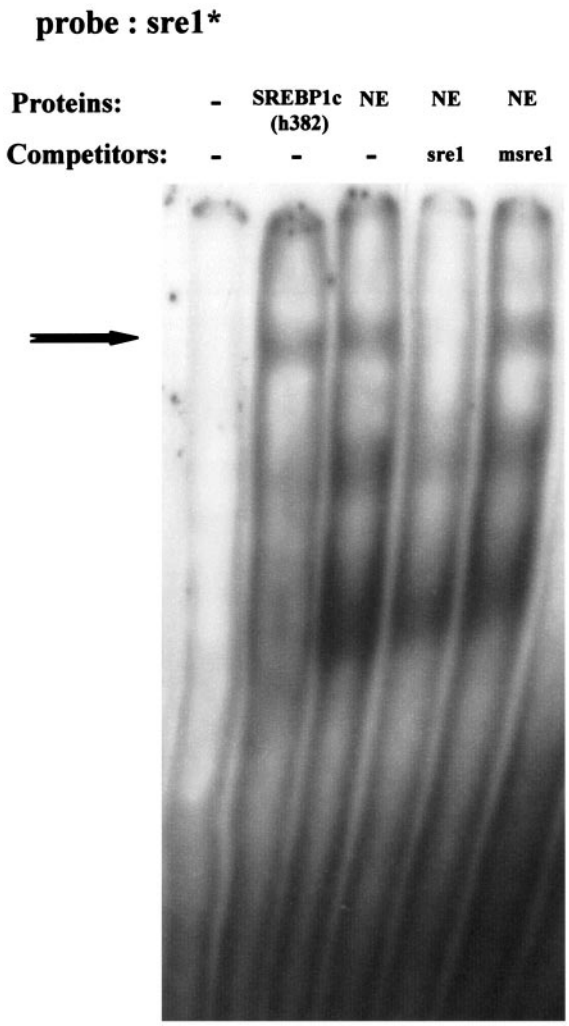

FIG. 4. Electrophoretic mobility shift analysis showing that the −309/−300 SRE of HKII promoter interacts with nuclear proteins prepared from human muscle cells. Electrophoretic mobility shift assays were performed as indicated in RESEARCH DESIGN AND METHODS, with 5′ end–labeled sre1 oligonucleotide in the presence of 15 μg of nuclear proteins prepared from differentiated myotubes. The arrow indicates the specific complex. NE, nuclear extract.

could not be verified using electrophoretic mobility shift assay.

Therefore, to demonstrate that SREBP-1 actually binds to the HKII promoter in human muscle cells, we used a ChIP assay. Chromatin from a large amount of differentiated myotubes (20 F25 Falcon flasks) was extracted after cross-linking with formaldehyde. SREBP-1 antibody was used to immunoprecipitate the fragmented chromatin, and then a specific region of the HKII promoter was amplified by PCR. Immunoprecipitation with an anti-RNA polymerase II antibody was performed in parallel as a positive control. Figure 5*A* shows the amplification products obtained using a p1/p2 primer set. The results clearly indicated that endogenous SREBP-1 binds to HKII promoter in human muscle cells. This result was confirmed with another set of primers (p3/p4) (data not shown). In contrast, no amplification was obtained when the PCR was performed with primers located in exon 2 of the HKII gene (~19 kb from the promoter region), indicating that the results were not attributable to contaminating genomic DNA (Fig. 5*B*).

Implication of SREBP-1 and −339/−330 SRE in the effect of insulin. Previous studies in hepatocytes (18) and in a mouse cell line (23) have demonstrated that insulin significantly increased the amount of nuclear SREBP-1c protein after a few hours of treatment and that this effect was blocked by inhibition of PI 3-kinase activity by wortmannin. Figure 6 clearly shows that incubation of human skeletal muscle cells for 6 h with 10^{-7} mol/l insulin increased the amount of SREBP-1c protein in both the membrane fraction (precursor form) and the nuclear protein extract (active form). Coincubation with wortmannin suppressed the effect of insulin. However, despite this increased amount of the nuclear form of SREBP-1c, it appeared that the interaction of SREBP-1 with the HKII promoter was not significantly modified, as assessed by the ChIP experiment (Fig. 5*C*). Only a slight increase in the intensity of the signal was observed in the presence of insulin. This likely reflected limitations in the quantitative aspect of the ChIP method. Indeed, clear differences in the level of the interaction are generally observed in hit-and-miss situations (24,25). Under our experimental conditions, SREBP-1c was already present in the nucleus of the muscle cells (Fig. 6), and there was a significant interaction between SREBP-1 and HKII promoter in the absence of insulin (Fig. 5). Changes in the intensity of the interaction was thus very difficult to ascertain.

To get more insight, we further verified that the SRE located at position −339/−330 was involved in the response of the HKII promoter to insulin using site-directed mutagenesis. The introduced mutations (GTCGCCCCAC replaced by ATTATCTCAC) have been previously shown to abolish the interaction of SREBP-1 with the SRE of the FAS promoter (22). In addition, we have observed that the sre1 probe bearing these mutations was unable to bind recombinant SREBP-1 proteins and did not compete with sre1 for the interaction with h382SREBP-1c (Fig. 3). The mutated promoter construct (pHKII-E2m) was transfected in human muscle cells, and Fig. 7 shows that mutation of the −339/−330 SRE site resulted in a complete loss of the effect of insulin. Basal luciferase activity was not significantly changed by the mutation of SRE. Thus, these data indicated that the SRE located at position −339/−330 is involved in the action of insulin on the expression of HKII gene in human muscle cells.

To further support the implication of SREBP-1c, we tested the effects of an overexpression of ADD1-403 (the constitutively active form of rodent SREBP-1c) on the activity of HKII promoter constructs in human myotubes. Figure 8 clearly shows that overexpression of ADD1-403 mimicked the effect of insulin on the constructs containing the SRE motif (pHKII-E1 and pHKII-E2), but not on pHKII-E4, which does not contain SRE. In addition, overexpression of ADD1-403 did not increase the promoter activity of the pHKII-E2m construct ($n = 2$, data not shown). To confirm the role of SREBP-1c in insulin action, we finally used a dominant-negative form of ADD1 (ADD1–403R) that did not bind sre1 in electrophoretic mobility shift assay (Fig. 3) and that has been shown to block SREBP-1 action by the formation of dimers with native SREBP-1 proteins (26). Figure 8 shows that overexpression of ADD1-403R completely abolished the effect of insulin on the pHKII-E2 promoter construct.

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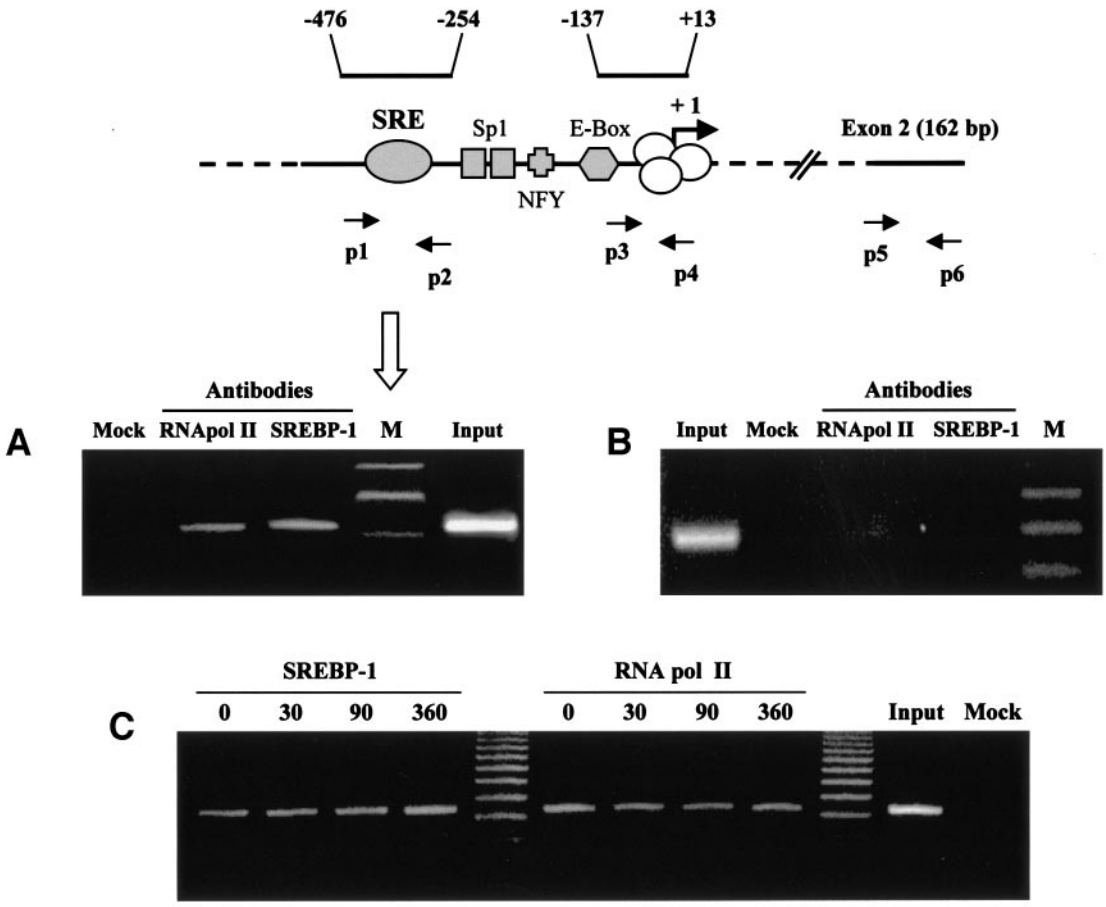

FIG. 5. Chromatin immunoprecipitation assay demonstrates the interaction of SREBP-1 protein with HKII gene promoter in human skeletal muscle cells. F25 culture flasks of differentiated myotubes were cultured in Dulbecco's modified Eagle's medium supplemented with 2% FCS for 7 days. To study insulin effects (*C*), cells were treated for 30, 90, and 360 min with 10^{-7} mol/l insulin. After cross-linking chromatin DNA and the interacting proteins, specific immunoprecipitations with anti-SREBP-1 or RNA polymerase (pol) II antibodies were performed as indicated in RESEARCH DESIGN AND METHODS. The PCR products were generated by the amplifications (35 cycles) of the HKII promoter region using the primers p1/p2 (*A* and *C*) and of the HKII exon 2 region (negative control) using primers p5/p6 (*B*). Amplification products were resolved in 3% agarose gel stained with ethidium bromide. M, marker lane.

DISCUSSION

HKII is the major isoform of HK in skeletal muscle, and its expression is induced by insulin in both rodent and human muscle (4–7). We have cloned the promoter region of the HKII gene and investigated insulin action in a primary culture of human skeletal muscle cells. Using luciferase reporter gene assay, we found a region of ~100 nucleotides (−369/−270) that conferred the transcriptional response to insulin. This region contains an SRE that interacted with recombinant active forms of SREBP-1c in electrophoretic mobility shift assay and binds endogenous SREBP-1 in ChIP experiments. Putative Sp1, NFY, and USF (E-box) binding motifs were located near this SRE, leading to a structure classically encountered in the region involved in the action of SREBPs on other promoters, such as those of FAS (24), LDL receptor, or ATP-citrate lyase genes (27). Overexpression of a constitutively active form of SREBP-1c (ADD1-403) reproduced insulin action on HKII promoter activity, whereas a dominant-negative form (ADD1-403R) completely prevented insulin response. In addition, insulin increased the amount of the nuclear form of SREBP-1c in the muscle cells. All together, these data indicated that SREBP-1c could be the mediator of the effect of insulin on the transcription of the human HKII gene in muscle.

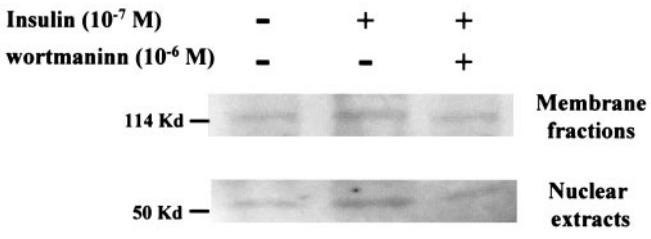

FIG. 6. Insulin induces the expression of SREBP-1c protein in skeletal muscle cells. Differentiated myotubes were treated or not treated for 6 h with insulin (10^{-7} mol/l). After cell lysis, the membrane fraction and the nuclear protein extract were prepared as indicated in RESEARCH DESIGN AND METHODS. SREBP-1c proteins (precursor and active forms) were detected by Western blotting using a mouse monoclonal antibody.

In rodent hepatocytes and adipose cell lines, it has been recently demonstrated that SREBP-1c is directly involved in the transcriptional effects of insulin on glucokinase- and lipogenesis-related genes (28,29). However, SREBP-1c is expressed at a much lower level in skeletal muscle than in liver or adipose tissue, thus questioning its possible role as an active transcription factor in muscle. Because both SREBP-1a and -1c are expressed in skeletal muscle (30) and because the two isoforms could efficiently bind clas-

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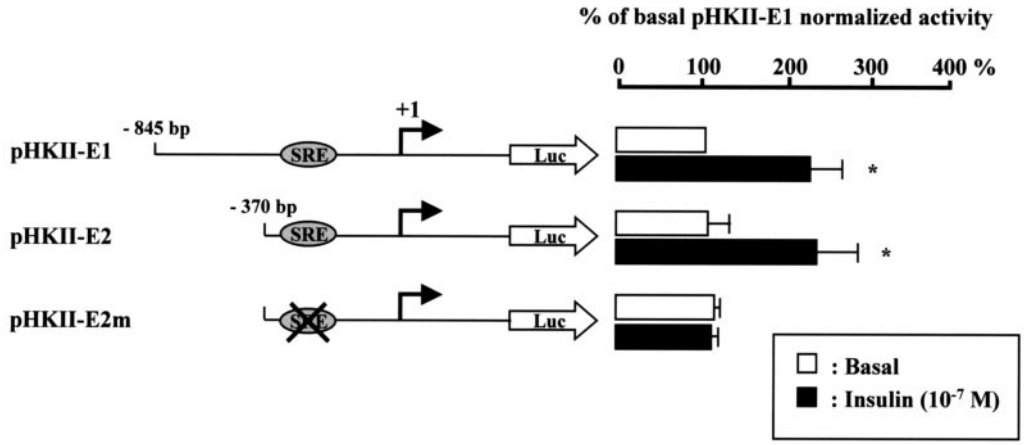

FIG. 7. Mutation of the −339/−330 SRE of the HKII promoter prevents insulin response. Human myotubes were transfected with a luciferase reporter gene driven by constructs of the human HKII promoter with and without mutation of the −339/−330 SRE. The mutation is indicated in Fig. 2. Cells were incubated for 24 h with or without insulin (10^{-7} mol/l). Data are expressed in terms of the basal luciferase activity of pHKII-E1 and are presented as the mean ± SE for four different transfection experiments. *$P < 0.05$ in the presence of insulin versus in the absence of insulin using a paired t test.

sical SREs (27), one could also not exclude the possibility that SREBP-1a instead of, or in addition to, SREBP-1c participates in the transcriptional effect of insulin on the HKII gene. Several lines of evidence do not yet support this hypothesis. Indeed, it has been found that SREBP-1c is more abundant than SREBP-1a in skeletal muscle (30). Moreover, insulin induced SREBP-1c expression at the mRNA (7,21) and protein levels in human muscle cells (Fig. 6). In contrast, it has been reported that insulin did not modify SREBP-1a expression (22,29,31). Furthermore, adenovirus-mediated overexpression of SREBP-1c mimicked the effects of insulin on the expression of glycolytic and lipogenic enzymes genes, including HKII, in cultured rat myotubes (32). All together, these data indicate that SREBP-1c is involved in the transcriptional effect of insulin on the HKII gene, and that it is likely to be one of the mediators of insulin action on gene expression in human skeletal muscle.

The mechanism by which insulin activates SREBP-1c is still a matter of debate. In different cell models, insulin increases the amount of both the precursor and the mature form of SREBP-1c (18,23,33), and this appeared to be a consequence of an induction of SREBP-1c mRNA expression (23). We found similar results in human muscle cells. Some reports suggested that insulin could induce the cleavage of the precursor form of SREBP-1c in the endoplasmic reticulum, leading to increased amounts of the mature form in the nucleus (33,34). However, this direct effect of insulin on the cleavage process was not observed in another study (18). It was also proposed that insulin

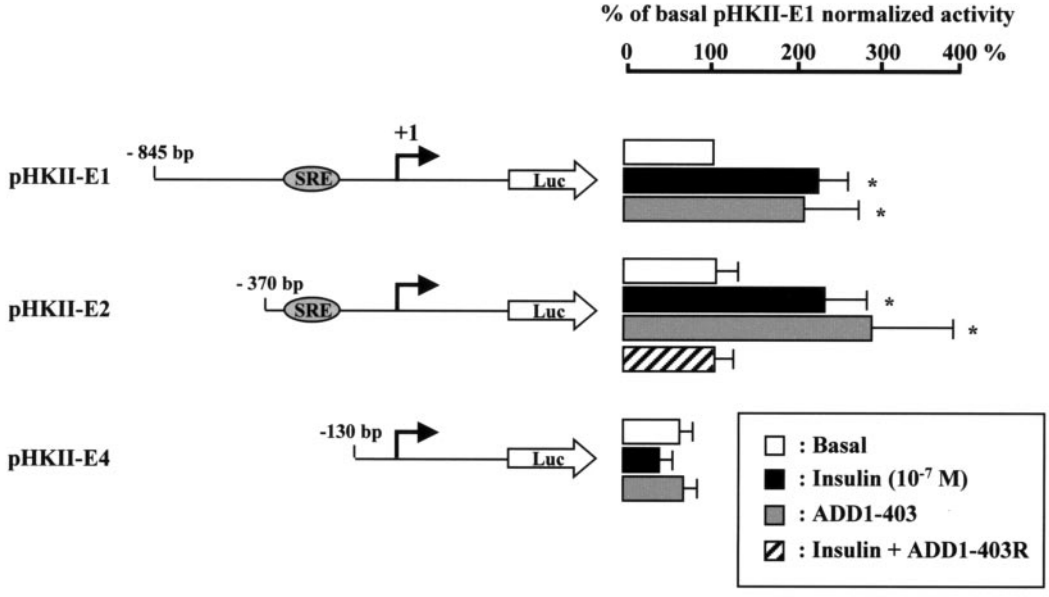

FIG. 8. Ectopic expression of ADD1 activates the promoter of the HKII gene in human muscle cells. Human skeletal muscle cells were cotransfected with HKII promoter–luciferase (Luc) reporter gene constructs and either the mature form (ADD1-403) or a dominant-negative form (ADD1-403R) of ADD1/SREBP-1c. ADD1-403 mimicked insulin, whereas ADD1-403R blocked insulin action. Data are expressed in terms of the basal luciferase activity of pHKII-E1 and are presented as the means ± SE for three different transfection experiments. *$P < 0.05$ in the presence of insulin or ADD1-403 versus in the absence of insulin using a paired t test.

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may directly activate the nuclear form of SREBP-1c by phosphorylation/dephosphorylation or by acting on a yet-unidentified partner (18). Additional experiments are thus required to better define the mechanism by which insulin and SREBP-1c affect HKII gene transcription.

In summary, the results of the present study demonstrate that SREBP-1c is involved in the effect of insulin on the transcription of the HKII gene in human myotubes. To our knowledge, this work provides the first identification of a transcription factor mediating the effect of insulin on gene expression in human muscle cells. We recently reported that ~500 genes are upregulated and ~300 are downregulated by insulin in vivo in human skeletal muscle (35) and that type 2 diabetes is associated with a marked alteration in the effect of insulin on gene expression (7). The characterization of the factors involved in the transcriptional action of insulin is thus a crucial step to better understand the mechanism of action of this hormone and the possible defects underlying insulin resistance.

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