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A Rare Missense Mutation in a Type 2 Diabetes Patient Decreases the Transcriptional Activity of Human Sterol Regulatory Element Binding Protein-1

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Contract grant sponsor Instituto de Salud Carlos III and Ministerio de Educación y Ciencia. Contract grant number: RCMN C03/08, SAF2003-01262;

Short Title: A Rare Missense Mutation Decreases the Transcriptional Activity of SREBP-1

Sterol regulatory element binding protein 1 (SREBP-1) transcription factors play a key role in energy homeostasis by regulating genes involved in both carbohydrate and lipid metabolism, and in adipocyte differentiation. The 5' end of the mRNA-encoding SREBP-1 exists in two forms, designated 1a and 1c. The divergence results from the use of two transcription start sites that produce two separate 5' exons, each of which is spliced to a common exon 2. Mutations in the sterol regulatory element binding protein gene (*SREBF*)-1 may contribute to insulin resistance states. However, the variants described to date do not affect the SREBP function. In this study, we investigated the functional consequences of a novel missense mutation common to both SREBP-1 isoforms identified in a Spanish Type 2 diabetic patient (c.677C>T, SREBP-1a p.T226M; c.605C>T, SREBP-1c p.T202M). Using reporter gene analysis and electrophoretic mobility shift assays, we found that this variant impaires the transcriptional activity and reduces DNA binding ability despite its comparable protein stability to the wild-type SREBP-1. This decreased activity impaires the expression of known downstream targets, such as the LDL receptor and fatty acid synthase genes. Our findings suggest that the threonine residue and/or surrounding region play an important role in the SREBP-1 function.".

KEY WORDS: SREBP-1, Type 2 diabetes, genetic variants, mutation.

INTRODUCTION

Sterol regulatory element binding proteins (SREBPs; OMIM #184756) are members of the basic helix-loop-helix (bHLH) family of transcription factors that directly activate the expression of genes involved in the cholesterol and fatty acid metabolism (Horton, et al., 2002). The mammalian genome encodes three SREBP isoforms: SREBP-1a and SREBP-1c are derived from a single gene on human chromosome 17p11.2 (*SREBF-1*) through the use of alternative promoters, and SREBP-2 is encoded by a separate gene on human chromosome 22q13 (*SREBF-2*) (Hua, et al., 1995). SREBP-1a is the predominant isoform in most cultured cell lines, spleen and intestine, whereas SREBP-1c predominates in the liver and most other tissues, such as muscle and adipose tissue (Shimomura, et al., 1997). SREBPs are synthesized as precursor forms bound to the endoplasmic reticulum and nuclear membrane. After proteolytic cleavage, active mature SREBPs enter the nucleus and activate the transcription of target genes by binding to either the sterol regulatory element (SRE), or to the E-box like sequences (Goldstein, et al., 2002). SREBP-2 cleavage is produced by cholesterol depletion, whereas insulin triggers SREBP-1c cleavage (Hegarty, et al., 2005). Under physiological conditions, SREBP-1c favours the fatty acid biosynthetic pathway and SREBP-2 favours cholesterogenesis (Horton, et al., 2002), whereas SREBP-1a seems to influence both the lipogenic and cholesterogenic pathways (Amemiya-Kudo, et al., 2002).

It has been proposed that SREBP-1c acts as a transcription factor, essential for the genomic actions of insulin on both carbohydrate and lipid metabolism (Foufelle and Ferre, 2002). Changes in this transcription factor activity might be a key to link insulin resistance with metabolic disorders at the molecular level. In fact Type 2 diabetes (OMIM #125853) and obesity (OMIM #601665), two common insulin-resistant states, are characterized by a decrease in the SREBP-1c mRNA levels in adipose tissue (Sewter, et al., 2002). In contrast, an elevated expression of SREBP-1c has been demonstrated in the liver of obese (ob/ob) mice, in insulin receptor substrate-2 deficient mice, and also in a transgenic mouse model of lipodystrophy (Shimomura, et al., 2000; Tobe, et al., 2001).
Recently, Laudes *et al.* have identified a common intronic single nucleotide polymorphism (SNP) between exons 18c and 19c, associated with the risk of diabetes and elevated plasma cholesterol levels (Laudes, et al., 2004). In addition, a case-control study performed in French obese and diabetic patients has shown that an SNP in exon 18c is associated with morbid obesity (Eberlé, et al., 2004). Therefore, variants in the *SREBF-1* gene may be responsible for insulin resistance, corroborating the relevant role of this transcription factor in metabolic disorders. Nevertheless, mutations affecting the SREBP-1 function have not yet been described. We have identified a novel missense mutation by searching for *SREBF*-1 mutations in Spanish subjects with Type 2 diabetes. Our study was designed to characterize the functional significance of this mutation in the pathogenesis of diabetes.

MATERIALS AND METHODS

Molecular genetic screening

Genomic DNA was subjected to an amplification of the *SREBF-1* gene using gene-specific primers designed with Primer express v 2.0" software (Applied Biosystems Foster City, CA. USA) according to published sequences (Hua, et al., 1995), to cover the coding regions of the complete SREBP-1 proteins and intron-exon boundaries (Table 1). PCR products were subjected to single-strand conformation polymorphism (SSCP) analysis, as previously described (Vedie, et al., 2001). Those products showing abnormal patterns were subsequently sequenced using the dideoxy chain termination method with the v2.0 BigDye terminator cycle sequencing kit and an ABIPrism 3100 DNA sequencer (Applied Biosystems Foster City, CA. USA).

Mutation nomenclature

All mutations are described as recommended, considering nucleotide +1 as the A of the first ATG translation initiation codon (Antonarakis, 1998; den Dunnen and Antonarakis, 2000); http://www.hgvs.org/mutnomen). When nucleotide position is indicated, cDNA SREBP-1a (GeneBank NM_004176.3) or SREBP-1c (GeneBank AK091131.1) sequences are used.

Construction of plasmids

Total RNA was isolated from frozen human adipose tissue using Tripure reagent (Roche Diagnostics, Manheim, Germany), and the first-strand cDNA was synthesized with MMLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega, Madison, WI. USA), using a gene-specific primer (5´-TGAGGTTCCAGAGGAGGCTACA-3´). SREBP-1c (aa 1-403) and SREBP-1a (aa 1-427) were cloned by PCR, using this cDNA as a template and the following primers: (i) SREBP-1c, forward primer 5'-GCGGCCGGTACCCGGAGCCATGGATTGCACTTTC-3', reverse primer 5'-GCGGCCAAGCTTTCATGGGGTCAGTGTGTCCTCCAC-3'; (ii) SREBP-1a, forward primer 5'-CTGCGCCATGGACGAGCCAC-3', same reverse primer. Cycle conditions were 95ºC 2 min, and 35 cycles of 95ºC 30s, 58ºC 30s and 72ºC 90s, followed by 72ºC 10min. PCR products were then ligated into pGEM-T-Easy Cloning Vector (Promega, Madison, WI. USA), and cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA. USA) after sequence conformation. Mutagenesis was performed by using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA. USA). The oligonucleotide used for in vitro mutagenesis was 5'-GCCCCCACGGCAGCCCCTGTAATGACCACTGTGACCTCGCAGATCC-3'. Truncated SREBP-1a (aminoacids 348-379) and -1c (aminoacids 324-355) were generated from pcDNA3-SREBP-1 expression vectors removing the corresponding sequences by digestion with endonuclease *Sml*I. The linearized plasmids were religated with T4 DNA ligase to generate Mut-1a and Mut-1c constructs.

Hepatocyte isolation, culture, and treatment with recombinant adenoviruses

The adenovirus vectors containing the mature SREBP-1c and c.605C>T (p.T202M) variant were constructed according to He et al (He, et al., 1998) as described previously (Foretz, et al., 1999). Hepatocytes were isolated from fed rats by the collagenase method (Casado, et al., 2001). Cell viability was assessed by the trypan blue exclusion test and was always higher than 85%. Hepatocytes were seeded at a density of 8×10^6 cells per dish (100-mm-diameter petri dishes) in medium DMEM containing 25 mmol/l glucose supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA. USA), 100 nM dexamethasone, 10 nM insulin and 100 nM triiodothyronine (T_3) (Sigma Chemica Co, St. Louis, MO. USA). After cell attachment (4h), the medium was replaced by a medium similar to the plating medium but containing 5 mmol/l glucose and free of hormones and the cells were cultured for 16h. Hepatocytes were then incubated for 2h with

adenoviruses at 5 multiplicity of infection (MOI). The medium was then replaced by fresh as described in the figure legends.

Table 1

Sequences of primers for molecular scanning of exon 1c to exon 19c of the human *SREBF-1* gene.

Exon	Amplicon	Forward primer	Reverse primer
1c	1	TCAACGGCTTCAAAAATCCG	GAGGCATTGTATAAAGGGCTGG
2	1	CCTCACTCACGCACCCTCTT	AATGGAGTGGGTGCAGGC
	2	CCTGCACCCACTCCATTGA	GTCCCCTCCCGCCACA
3	1	GCAGCCCCATGCTTTATTTCT	GCTTGGCTGTAAGCTGTGTGTCT
4	1	TTACAGCCAAGCCTCTCCCA	CTTATGCCCTGCCCACGT
5	1	CACATGCCCACCTGGTAACA	TGCAGGCCTCTCCACACC
6	1	CCACACCGTGCAGCTGAATA	GAGGTGGAGCAAGGCTAGAGAAG
7	1	TGTTCCTTTCGGCCCACA	CAGGGCCCAACCTTGCT
8	1	ACCCCACCAGGCAAAGC	CCGGTCCCACCTCTGCT
9	1	AGCTTTCCCTCTGTCCATAGATG	GGAACAAGGGTTGACACCCA
10	1	GCTGCTCTGACCTCACTTTTCTC	TGAGGTTCCAGAGGAGGCTACA
	2	GTAGCCTCCTCTGGAACCTCATC	CCACGCTCAGTCCTACCCAT
11	1	TGCCTGTGCTCCACCCA	CCCAACCCACTCACTGTCAGA
12	1	CAGCGCTTCTTCCTGAGCAG	AAGAGAGCACCTGGGTTCCC
13	1	TCTCCACAGTGGACCCCCT	ACTTACTTGTCCCCATCAGCTGA
14	1	TAGGACCCCTGCCATCTGTT	GGAGCTGAGAAGGGAGCCA
15	1	TCCCTCTTCTGCAGGCGTA	AGGAACCTGCCGTGCACT
16	1	ACTGGTCTGACTGGCACTCTTCT	TCACCTTGTCAATGGAGCTGC
17	1	CAGGCCGTGCAGCTGTT	CACTCACCCTCCGCATGG
18c	1	TGTCTTCAGCTGATGGATGTGC	CTAACAAACAAACATCGGGAAGAG
19c	1	GCTGTGTCAACCCTTAGGCG	GAGGAGGCTTCTTTGCTGTGA
	2	CTGCCGCCACACTGGAG	GAGTAAAAAACAGTCATTGCATTCAGA

Cell culture and luciferase assay

COS-7 cells were grown in DMEM containing 25 mmol/l glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin supplemented with 10% fetal bovine serum (FBS). They were maintained at 37ºC in a humidified atmosphere containing 5% CO_2, and were passaged by trypsinization with trypsin-EDTA (0.2-0.5 mol/l) on a weekly basis.

The plasmid constructs were transfected into COS-7 cells using Lipofectamine plus reagent (Invitrogen, Carlsbad, CA. USA) in the presence of 10% FBS. 750 ng of either wild-type or p.T202M SREBP-1c expression vectors were transfected along with 1500 ng of Firefly luciferase reporter construct containing rat liver type glucokinase promoter (pRGKL-1448 or pRGKL-1448mab; kindly provided by Dr. Ahn, Yonsei University College of Medicine, Korea) or empty vector (pGL3 basic), and 750 ng of pGFPN1 vector as an internal control (BD Biosciences, San José, CA. USA). Transactivation activities were measured after 24h using a luciferase reporter gene assay (Roche Diagnostics, Manheim, Germany) in a Wallac 1420 VICTOR luminometer. Firefly luciferase activity values were normalized to those of GFP fluorescence.

Electrophoretic mobility shift assay (EMSA)

Wild-type, p.T202M and p.T226M proteins were synthesized from 650 ng of pcDNA3 expression vectors using TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI. USA). *In vitro* translated proteins were

incubated with ^{32}P-labelled oligonucleotide containing either the consensus SRE motif (5´-GATCCTGATCACCCCACTGAGGAG-3´), or the -65 E box of fatty acid synthase (*FAS)* gene (5´-TCAGCCCATGTGGCGTGGCCGC-3´) in a 20µl reaction mixture, as previously described (Casado, et al., 2001). DNA-protein complexes were resolved on a 6% polyacrylamide gel using 0.5X TBE buffer. PhosphorImager analysis (FLA5000, Fuji Photo Film (Europe), Düsseldorf, Germany.) was used to quantify the DNA-protein complexes.

Western-blot analysis and Real-time quantitative PCR

Chang liver cells were transfected with 4 µg of pcDNA-SREBP-1 expression vectors (wild-type or mutants) or with pcDNA empty vector using Lipofectamine plus reagent (Invitrogen, Carlsbad, CA. USA) for 3h. After a further 3h or 9h, cells were lysed in an ice-cold buffer containing 50 mmol/l Hepes (pH 7.5), 150 mmol/l NaCl, 1 mmol/l dithiothreitol, 0.1 mmol/l orthovanadate, 10 mmol/l beta-glycerophosphate, 2.5 mmol/l EGTA and 10% glycerol, supplemented with protease inhibitor CompleteTM Mini-mixture (Roche Diagnostics, Manheim, Germany). Protein concentration in cell lysates was determined by the Bradford method (Bio-Rad, Hercules, CA., USA). Protein analysis were carried out by western blot as previously described (Casado, et al., 2001) using antibodies to SREBP-1 (BD Biosciences Pharmingen. San Jose, CA. USA). Anti-actin antiserum (Sigma Chemica Co, St. Louis, MO. USA) was used as an internal control. The levels of protein were quantified using an Image Reader Las3000 and Image gauge v4.0 software (Fuji Photo Film (Europe), Düsseldorf, Germany).

Total RNA was isolated from transfected Chang liver cells or cultured hepatocytes by using Tripure reagent (Roche Diagnostics, Manheim, Germany). First strand cDNA was synthesized from 1 µg of total RNA using random hexamer and expand reverse transcriptase (Roche Diagnostics, Manheim, Germany). cDNA was used as a template for real-time PCR in the GeneAmp 5700 Sequence Detection System (Applied Biosystem Foster City, Ca. USA.). The relative amount of each mRNA was calculated using the second derivative comparative Ct method. A control cDNA (empty vector sample) was used as an interplate calibrator and the variability in the initial quantities of cDNA was normalized by using GAPDH as an endogenous control. The PCR conditions were 1 cycle at 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s and at 60ºC for 1 min. The sets of primer and Taqman probes used to analyze cDNA from Chang liver cells were proprietary to Applied Biosystems (Assays-on-demand gene expression product; Applied Biosystems Foster City, CA. USA). For the expression of rat glucokinase in cultured hepatocytes, Real-time PCR was performed using SYBR Green I Master Mix and gene-specific primers designed by using Primer Express software (Applied Biosystems Foster City, CA. USA). Primers used were as follow: rat GK-sense, 5'- CCGTTTCGTGTCACAAGTGG -3'; GK-antisense, 5'-AATGTCGCAGTCGGTGACAG-3'; rat GAPDH-sense, 5'-CAAGGTCATCCATGACAACTTTG-3'; GADPH-antisense, 5'-CTGAGTGGCAGTGATGGCAT-3'.

Confocal immunofluorescence.

Cells grown on glass coverslips were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and 100% methanol (precooled at -20ºC) for 20 min, and washed in PBS (2x, for 5 min). Slides were incubated with SREBP-1 primary antibody at 1:50 dilution for 16h at 4ºC, washed in PBS (5x, 5 min), and incubated with the appropriate secondary antibody coupled to fluorescein for 50 min. TO-PRO-3 (Molecular probes) was used for DNA staining. Confocal images were obtained with a Leica TCS SP2 Spectral microscope and a 40x/1.25 NA oil objective (Leica Microsystems, Wetzlar, Germany).

Statistical analysis.

Data show the means ± SEM of three or four experiments. The statistical significance was estimated with the Student's two-tailed t-test for unpaired observations. A p value of less than 0.05 was considered to be significant.

RESULTS

We identified a novel missense mutation c.605C>T (p.T202M) by searching for *SREBF*-1 mutations in Spanish subjects with Type 2 diabetes. The allelic variant was found in a single patient, a 48-year old male with hypertension (175/90 mm Hg), obesity (BMI=30.3 Kg/m^2), hypercholesterolemia (T-chol 250 mg/dl), and an elevated total cholesterol/HDL-cholesterol ratio (250 mg/dl/45 mg/dl=5.55). Since the relatives of this patient were not available for testing, we performed *in vitro* experiments to evaluate the impact of the mutation on the SREBP function, thus verifying its possible implication in the development of diabetes in the affected subject.

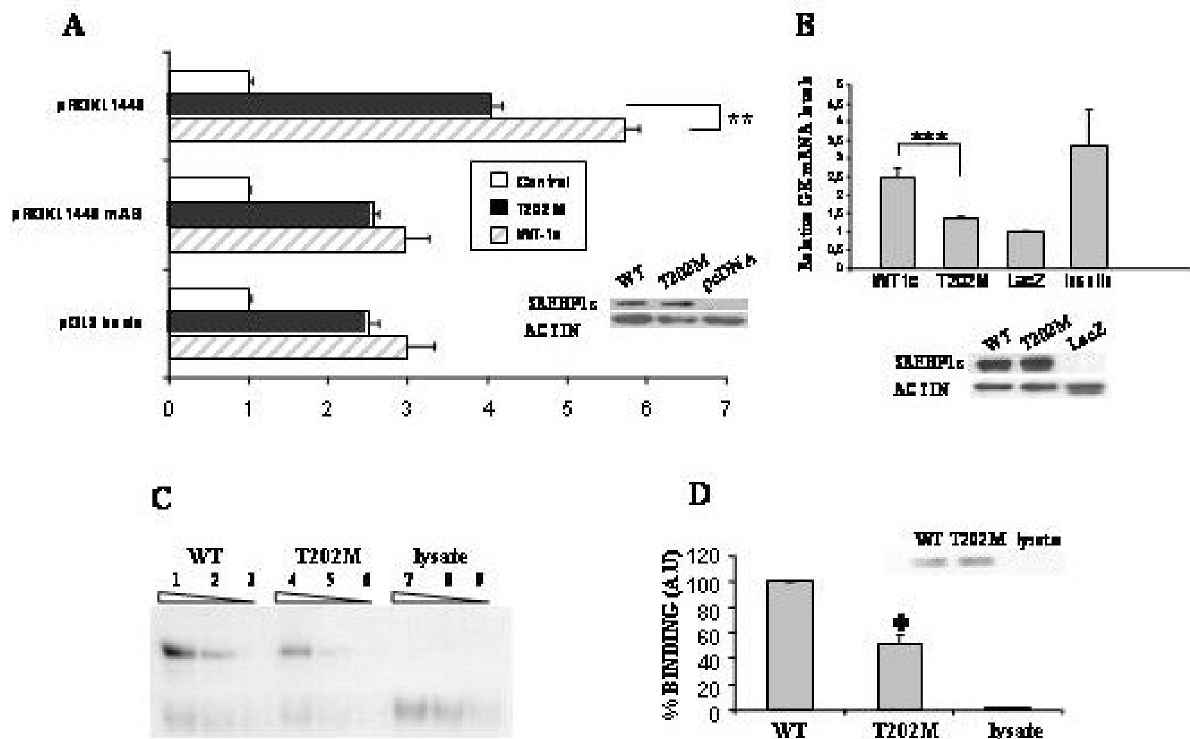

Fig. 1. Transactivation activity and DNA binding properties of c.605C>T (p.T202M) mutation. A. COS-7 cells were transiently co-transfected with expression vectors for SREBP-1c (WT or p.T202M), a reporter plasmid carrying the coding sequence of firefly luciferase under the control of glucokinase promoter (pRGKL-1448), or a SRE-truncated mutant (pRGKL-1448mab), and pGFPN1 expression vector coding GFP protein, as an internal control. Cells were harvested 24h post-transfection, and luciferase activity and GFP fluorescence were measured as indicated in Material and Methods. Data are presented as luciferase activity normalized to GFP fluorescence, and represent the average of three independent transfection experiments and two luciferase activity determinations for each. Normalized luciferase activities are expressed as a fold increase relative to the basal activity in the absence of the SREBP-1c expression vector (control) (**$p< 0.01$). **B**. Hepatocytes were cultured for 16h after plating in the presence of 5 mM glucose. Cells were then incubated with 5mM glucose either with or without 100 nM insulin or without insulin and with 5 MOI of adenoviral vectors. After 18h, total RNAs were extracted and analyzed for the expression of GK. SREBP-1 protein expression in cell extracts was detected by Western blot. A representative experiment is shown beneath the chart. The fold induction value for quadruplicate wells was averaged, and data are presented as mean ± SEM. (***$p< 0.001$). **C.** An electromobility gel shift assay was performed with in vitro-translated SREBP-1c wild-type (WT) and T202M mutant. Different amounts of SREBP [6 µl (lanes 1, 4, 10, 11 and 12), 4 µl (lanes 2 and 5) and 2 µl (lanes 3 and 6)] and 6, 4 and 2 µl of control reticulocyte lysate (lanes 7, 8 and 9, respectively) were incubated at 4ºC for 30 min with radiolabelled double-stranded DNA fragment containing an SREBP *cis* element, as described in "experimental procedures". One representative of three independent experiments is shown. **D** Quantification of DNA binding: results are presented as a percent of control (SREBP-1 WT) after normalisation relative to their respective levels of translated proteins determined by Western blot (representative experiment included in the graph). All data represent mean ± SEM. Statistical comparison among groups was carried out using the Student´s t test. *p values < 0.05 were considered to be significant.

We first studied its ability to activate transcription using a promoter reporter gene assay. We observed that compared to the wild-type construct, the p.T202M mutation significantly reduced the ability of SREBP-1c to

activate the glucokinase (GK) promoter, whose expression is SREBP-1 dependent (Foretz, et al., 1999; Kim, et al., 2004). Neither of the two proteins was able to activate a construct containing double mutations in the two functional SRE motifs identified in the rat GK promoter (see pRGKL 1148mAb vs pGL3 basic), indicating that the decrease of activity observed with the mutation was SREBP-specific through its SRE elements present in the target gene promoter (Fig 1A). As shown in Fig.1A, wild-type and p.T202M proteins were significantly expressed in cells, as determined by Western blot.
To test the effect of SREBP-1c directly on endogenous gene expression rather than on promoter reporter gene assay, we infected cultured rat hepatocytes with wild-type and p.T202M proteins by using adenoviral vectors. Rat hepatocytes cultured in presence of insulin or infected with wild-type SREBP-1c adenoviral vector expressed glucokinase mRNA. Adenovirus-mediated expression of the p.T202M SREBP-1c mutant reduced significantly the expression of glucokinase compared to the induction due to wild-type SREBP-1c or insulin. SREBP-1c proteins were equally expressed after adenoviral infection (Fig. 1B). This result confirms the lower activity of the SREBP-1c variant.

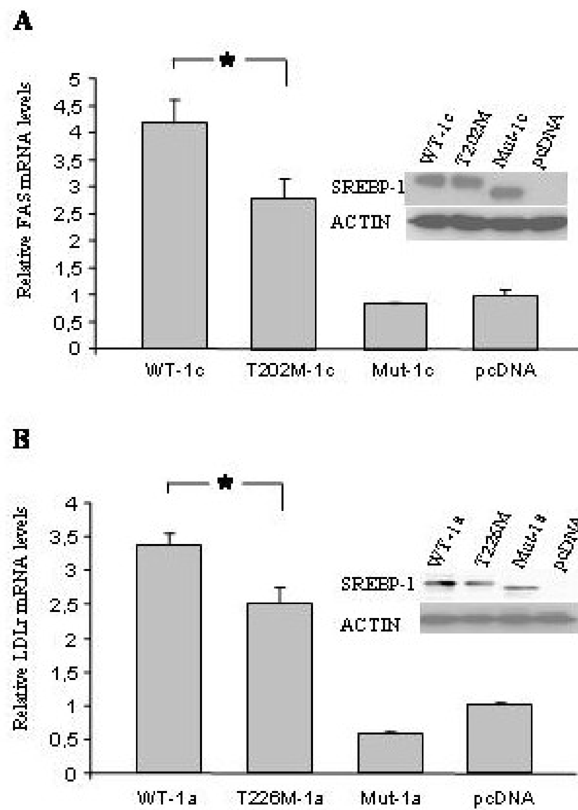

Fig 2. Effect of the SREBP-1 mutants on the target gene expression. Chang liver cells were transfected with pcDNA3-SREBP-1a and -1c (wild type or p.T202M and p.T226M mutants), pcDNA3-SREBP Mut-1a or -1c (negative control) or with an empty vector (pcDNA3), as described under "materials and methods", and quantitative real-time PCR analysis of the target genes expression was performed. Fatty acid synthase was used as a target gene to monitor SREBP-1c activity (panel A), and the LDL-receptor (LDLr) was used as a target gene to monitor SREBP-1a activity (panel B). The fold induction value for quadruplicate wells was averaged, and data are presented as mean ± SEM. (*$p < 0.05$). Cell protein lysates were analysed by immunoblotting with an anti-SREBP-1 antibody to examine the protein expression level. Expression of the housekeeping gene actin is shown in the bottom panel and served as a loading control.

We further investigated whether this loss of activity was due to a reduced SREBP DNA binding ability. EMSA experiments were performed using *in vitro* translated mature wild-type and p.T202M mutant, and radiolabelled

double-stranded DNA fragment containing a known SREBP *cis* element (Brown and Goldstein, 1997). All proteins were able to bind to this oligonucleotide, as shown by the retarded complex observed in all cases when compared to the nonprogrammed reticulocyte lysate. However, the p.T202M mutant showed a lower binding efficiency when compared with wild-type SREBP-1c (Fig. 1C, 1D). As this diminished binding could be due to a lower efficiency in the translation of T202M-SREBP-1c vector, we verified by western blot the levels of translated proteins, which were comparable for all vectors (Fig. 1D). Similar binding results were obtained when we used a radiolabelled double-stranded oligonucleotide corresponding to the -65 E box sequence of the proximal promoter of the fatty acid synthase (*FAS*) gene (data not shown). c.605C>T (p.T202M) is localized in exon 3 of the *SREBF-1* gene, a region common to both SREBP-1a and -1c isoforms. The experiments were repeated with SREBP-1a expression vectors which carried the c.677C>T (p.T226M) mutation and similar results were obtained (data not shown).

In order to analyze whether the mutation altered the expression of SREBP-1c target genes in human cells, FAS mRNA levels were estimated by real-time PCR in Chang liver cells transfected with expression vectors containing either wild-type or p.T202M SREBP-1c. As Fig.2A shows, the induction of FAS mRNA levels was significantly reduced by the p.T202M variant, in spite of the similar expression levels of proteins. As SREBP-1a also influences the expression of cholesterogenic enzymes, we verified the effect of p.T226M SREBP-1a on the LDL receptor expression. We also observed a slight decrease in the LDL receptor mRNA levels (Fig. 2B). As negative control, we analyzed the effect of a non-functional SREBP-1 (Mut-1a, -1c) containing a mutation which abolished the DNA binding and dimerization. This construction abolished the SREBP-1 dependent-gene expression (Figs. 2).

We verified the correct subcellular localization of proteins by confocal immunofluorescence. A homogenous nuclear stain of both SREBP-1c and p.T202M proteins was observed (Fig. 3), indicating that the expressed proteins were mature forms that entered the nucleus to activate the target genes.

DISCUSSION

In this study we describe the first SREBP-1 mutation found in a Type 2 diabetic patient that modifies the function of the protein, diminishing its DNA binding efficiency and its ability to activate the transcription of target genes. Unfortunately, family data was not available for the cohort studies.

SREBP-1c has a pivotal role in glucose homeostasis by transducing the insulin effects on the hepatic gene expression. The overexpression of SREBP-1c, specifically in the liver of diabetic mice, leads to a marked decrease in blood glucose, due in part to an increase in the expression of hepatic glucokinase, which in turn enhances hepatic glycolysis and lipogenesis (Becard, et al., 2001). In contrast, a loss of SREBP-1c function should lead to insulin resistance. There is a tendency in SREBP-1c knockout mice for higher basal plasma glucose and higher glycemia during a carbohydrate refeeding period (Liang, et al., 2002). However, recent studies demonstrate that a high level of SREBP-1c results in further hepatic insulin resistance through a suppression of the IRS-2 expression, as well as a formation of fatty liver and a production of triglyceride-rich VLDL, that could be converted to atherogenic remnant particles (Ide, et al., 2004). Therefore, it is difficult to predict whether the lower efficiency of the c.605C>T (p.T202M) mutation would play a role in the development of diabetes in the affected subject if our results were exclusively taken into consideration. A large-scale population genetics and studies of pedigrees are of relevance.

Transcriptionally active mature SREBPs share a similar tripartite structure, consisting of an acidic NH2-terminal sequence, a domain rich in proline, serine, glycine and glutamine residues, and a bHLH-LZ domain allowing dimerization, nuclear import and DNA binding (Brown and Goldstein, 1997) The threonine residue involved in the missense mutation is localised in a low complexity region placed between the proline/serine rich domain and the bHLH-LZ domain. The low complexity regions are often mosaics of a small number of amino acids. These regions have been shown to be functionally important in some proteins, but they are generally not very well understood. We made a multiple sequence alignments as a tool to help categorise this mutation as potentially disease related change (Abkevich, et al., 2004). The threonine is invariant in distantly related species as Tetraodon or *C. elegans*, which could be indicative of a functional role.

Unlike other bHLH-LZ transcription factors, SREBPs contain a tyrosine residue in their basic domain, allowing them to bind to both E-boxes and SRE sequences (Kim, et al., 1995). We report the first evidence that mutations outside the b-HLH-LZ domain affect SREBP-1 DNA binding. This phenomenon of intramolecular inhibition of DNA binding could be caused by destabilizing protein-DNA complexes or by conformational changes, as described for other transcription factors, such as Ets-1 (Jonsen, et al., 1996).

Besides sterol or insulin-dependent cleavage, SREBPs are regulated by additional steps at a post-translational level. It has been reported that SREBPs could be modified in different residues by phosphorylation, acetylation, ubiquitylation or sumoylation (Giandomenico, et al., 2003; Hirano, et al., 2003; Roth, et al., 2000; Sundqvist and

Ericsson, 2003). This mutation could affect post-translational modifications of SREBP-1 but this needs more investigation to conclude.
To summarize, we have identified a novel functional mutation in the *SREBF*-1 gene which alters the binding and transcriptional activity of SREBP-1, suggesting that the threonine 202, and/or the surrounding region, play an important role in the SREBP-1 function. The advance in identifying key residues in the SREBP-1 function is important to understand the role of this transcription factor in pathophysiologies, such as diabetes and obesity, as well as its potential use as a therapeutic target.

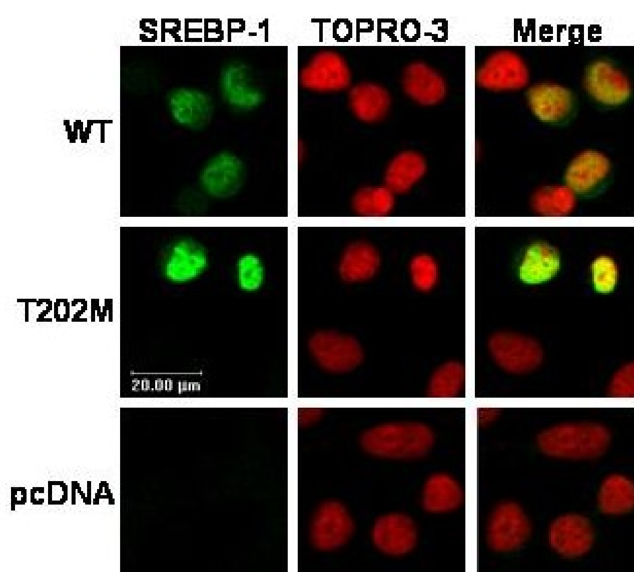

Fig 3. Immunocytochemical analysis of the SREBP-1 subcellular localization. Immunocytochemistry images shown represent Chang Liver cells that have been transfected with mature SREBP-1 (WT or p.T202M), or with empty pcDNA3 expression vectors. SREBP-1c-positive cells (green), nuclear staining (red) or merged images are shown. Scale bar, 20 μm. Original magnification, 40×.

ACKNOWLEDGEMENTS

We thank Dr. J. Perez-Tur, Dr. F. Palau and co-workers for the indispensable help with the SSCP work, and Dr. P. Martin-Sanz and Dr. P. Sanz for helpful discussion This study was supported by grants from Instituto de Salud Carlos III, Red de Centros (RCMN) C03/08, and from Ministerio de Educación y Ciencia (SAF2003-01262). S.V. is supported by a fellowship from Consejo Superior de Investigaciones Científicas I3P-BPD2001-1.

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