Insulin and Sterol-regulatory Element-binding Protein-1c (SREBP-1c) Regulation of Gene Expression in 3T3-L1 Adipocytes

IDENTIFICATION OF CCAAT/ENHANCER-BINDING PROTEIN β AS AN SREBP-1C TARGET*

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We evaluated the hypothesis of sterol-regulatory element-binding protein (SREBP)-1c being a general mediator of the transcriptional effects of insulin, with a focus on adipocytes, in which insulin profoundly influences specific gene expression. Using real time quantitative reverse transcriptase-PCR to monitor changes in the expression of about 50 genes that cover a wide range of adipocyte functions, we have compared the impact of insulin treatment with that of adenoviral overexpression of either dominant positive or dominant negative SREBP-1c mutants in 3T3-L1 adipocytes. As expected, insulin up-regulated, dominant positive stimulates, and dominant negative decreased previously characterized direct SREBP targets (FAS, SCD-1, and low density lipoprotein receptor). We also identified three novel SREBP-1c transcriptional targets in adipocytes, which were confirmed by run-on assays: plasminogen activator inhibitor 1, CCAAT/enhancer-binding protein δ (C/EBPδ), and C/EBPβ. Because most insulin-regulated genes were also modulated by SREBP-1c mutants, our data establish that 1) SREBP-1c is an important mediator of insulin transcriptional effects in adipocytes, and 2) C/EBPβ is under the direct control of SREBP-1c, as demonstrated by the ability of SREBP-1c to activate the transcription from C/EBPβ promoter through canonical SREBP binding sites. Thus, some of the effects of insulin and/or SREBP-1c in mature fat cells might require C/EBPβ or C/EBPδ as transcriptional relays.

Insulin is the main anabolic hormone in mammals and exerts its effects in liver, adipose tissue, and skeletal and cardiac muscle via the insulin receptor (for a review, see Ref. 1). The cellular mechanism underlying its action on carbohydrate, lipid, and protein metabolism has been the center of major interest for many years. Active research has led to the identification of the major steps of the insulin signal transduction pathway. These include a family of soluble scaffolding molecules, known as insulin receptor substrates, which initiate downstream signaling cascades involving the phosphatidylinositol 3-kinase/Akt pathway and the mitogen-activated protein kinase pathway (for reviews, see Refs. 1 and 2). In this cascade, rapid changes in the state of protein phosphorylation ultimately mediate many important actions of insulin (e.g. glucose transport, glycogen synthesis, lipogenesis, and antilipolysis).

It is also well known that, alongside these rapid nongenomic effects, important changes in gene expression play critical roles in insulin action in insulin-sensitive tissues (3). The transcriptional effects of insulin and the mechanisms by which insulin can relay signal to the nucleus have remained largely unknown until recently. As described (4), new light was shed by the identification of SREBP-1c as a transcription factor capable of mediating some of the effects of the hormone on previously identified insulin target genes. Indeed, SREBP-1c was shown not only to regulate the expression of key genes of glucose, fatty acid, and triglyceride metabolism in fibroblasts, adipocytes, hepatocytes, and the livers of transgenic mice (5–7) but also to be able to substitute to insulin in inducing transcription of known insulin target genes like glucokinase or FAS in hepatocytes (8). SREBP-1c is particularly abundant in the adipose tissue and the liver, both of which are insulin-sensitive and display quite a restricted expression pattern compared with the ubiquitously expressed SREBP-2, the other SREBP isoform that is encoded by a separate gene (9). In agreement with their distinct expression pattern and regulation, SREBP-1c and SREBP-2 can also be distinguished in vivo by their ability to target different genes. Indeed, SREBP-1c and SREBP-2 assume different functions, SREBP-2 being more selective for activating genes involved in cholesterol homeostasis (reviewed in Ref. 10), whereas SREBP-1c actions are focused on lipid synthesis and glucose metabolism. From these studies, SREBP-1c thus appears as a strong candidate to be a general mediator of the metabolic actions of insulin via the regulation of gene expression.

The aim of the present study was to document further this hypothesis with a focus on adipocytes, in which specific gene

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1 The abbreviations used are: SREBP, sterol-regulatory element-binding protein; Ad, adenovirus; MOI, multiplicity of infection (i.e. plaque-forming units per cell); DN, dominant negative; DP, dominant positive; SRE, sterol-regulatory element; HMG, hydroxymethylglutaryl; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/enhancer-binding protein; RT-PCR, reverse transcriptase-PCR; LDL, low density lipoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKB, protein kinase B; GFP, green fluorescent protein; PAI, plasminogen activator inhibitor.
expression is profoundly influenced by insulin. In the context of the adipose cell, several transcription factors that play interconnected roles ultimately determine the fully differentiated adipocyte gene expression profile. Among these factors is SREBP-1c, known also as ADD-1 (for adipocyte determination and differentiation factor-1 (11)), a member of the basic helix-loop-helix-leucine zipper family of transcription factors. Other important adipocyte transcriptional regulators include the fatty acid derivative-activated nuclear receptor zinc finger peroxisome proliferator-activated receptor γ (PPARγ) and several members of the basic leucine zipper family of CAAT/enhancer binding proteins (C/EBPs) (reviewed in Ref. 12). In particular, C/EBPβ and C/EBPδ, when induced by appropriate stimuli, can initiate a transcriptional cascade that culminates in the induction of PPARγ and C/EBPα and the activation of the adipogenic program.

In this study, we have used mature 3T3-L1 adipocytes to investigate the impact of the overexpression of mutant SREBP-1c isoforms, either dominant positive or dominant negative, on a panel of 50 adipocyte-specific genes. The latter were selected to cover a number of aspects of key fat cell functions such as lipid storage, lipolysis, glucose metabolism, energy expenditure, adipocyte-gene transcription factors, and adipocyte-derived secreted products. Our results show that genes that were up-regulated by the dominant positive and down-regulated by dominant negative SREBP-1c forms were also up-regulated by insulin, confirming that SREBP-1c is a major factor underlying the transcriptional effect of insulin. Moreover, we found out that SREBP-1c specifically mediated insulin action on some adipocyte genes, such as PAI-1, and the β and δ C/EBP isoforms, which were known as insulin-sensitive but previously unrecognized as SREBP-1c targets. Finally, we provide evidence that SREBP-1c can directly transactivate the C/EBPβ promoter through canonical C/EBP binding sites. Moreover, those sites map the insulin response region of the C/EBPβ promoter. Thus, this study demonstrates the existence of an insulin-SREBP-1c-C/EBPβ axis in adipocytes and suggests that a transcriptional cascade might be initiated by SREBP-1c to mediate insulin effects on fully differentiated adipocyte gene regulation. MATERI A LS AND METHODS

Preparation of Recombinant Adenoviruses—The adenovirus vector containing the transcriptionally active dominant positive (DP) amino-terminal fragment (amino acids 1–403) of rat SREBP-1c, Ad.SREBP-1c DP, was constructed as previously described (8) with homologous recombination in BJ5183 bacteria using the shuttle vector pAdTrack-CMV containing the green fluorescent protein (GFP) (13). The recombinant adenovirus containing the dominant negative form of rat SREBP-1c, Ad.SREBP-1c DN, was described elsewhere (14). Both Ad.SREBP-1c DP and Ad.SREBP-1c DN were under control of a cytomegalovirus promoter. The adenovirus vector containing the major late promoter with no exogenous gene (Ad.null) was used as control. The adenoviral vectors were propagated in the HEK 293 cell line, purified by cesium chloride density centrifugation, and stored at −80°C until use. The efficiency of infection in 3T3-L1 adipocytes was assessed by visualizing GFP expression using a fluorescence microscope (Eclipse E800, Nikon). Measurements of SREBP target gene expression were also performed using various MOI (from 10 to 50) and various postinfection times from 24 to 72 h. Experiments were performed 4–6 times. Various tested conditions were Ad.SREBP-1c DP with no insulin; Ad.SREBP-1c DN with insulin (100 nM); Ad.null with insulin (100 nM); and Ad.null with no insulin. 3T3-L1 Cell Culture—3T3-L1 cells (ATCC number CL-173) were grown in 6-cm diameter dishes and differentiated at 37 °C in an atmosphere of air/CO2 (90:10, v/v) in Dulbecco’s modified Eagle’s medium (Invitrogen) with 4.5 g/liter glucose, 10% fetal calf serum, penicillin/streptomycin (50 units penicillin/50 μg of streptomycin per ml of medium). Two days after reaching confluence, cells were induced into differentiation with a 2-day incubation in Dulbecco’s modified Eagle’s medium, 10% fetal-calf serum containing insulin (1 μg/ml), dexamethasone (0.25 μM), and isobutylmethylxanthine (0.1 mM) (all from Sigma). Then preadipocytes were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum supplemented with insulin (1 μg/ml). After 10 days, when adipocytes have accumulated numerous lipid droplets as judged by Oil Red O staining, cells were plated for 16–18 h in a reduced glucose-containing Dulbecco’s modified Eagle’s medium consisting of Dulbecco’s modified Eagle’s medium (12:1, v/v), 4.5 g/liter glucose, glutamine, penicillin/streptomycin, free fatty acid bovine serum albumin (5%) (Sigma), in the absence or in the presence of insulin (100 nM), and then treated for various times (24–72 h) at an MOI of 10–500 with the different recombinant adenoviruses.

RNA Preparation and Real Time Quantitative RT-PCR—Total RNA was extracted as described in Materials and Methods. cDNA was synthesized from 5 μg of total RNA in 20 μl using random hexamers and murine leukemia virus reverse transcriptase (Invitrogen). The design of primers was done using either Primer Express (Applied Biosystems) or Oligo (MedProbe, Olso, Norway) software. Real time quantitative RT-PCR analyses for the genes described in Table I were performed starting with 50 ng of reverse transcribed total RNA (diluted in 5 μl of 1× Sybr Green buffer), with a 200 ng concentration of both sense and antisense primers (Genset) in a final volume of 25 μl using the Sybr Green PCR core reagents in an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). Fluorescence is generated after laser excitation by bound Sybr Green to double-stranded DNA. Because we used Sybr Green in measurements of amplification-associated fluorescence for real time quantitative RT-PCR, it is important to verify that generated fluorescence was not overestimated by contamination resulting from residual genomic DNA amplification (using controls without reverse transcriptase) and/or from primer dimers formation (controls with no DNA template nor reverse transcriptase). RT-PCR products were also analyzed on ethidium bromide-stained agarose to ensure that a single amplification of the expected size was indeed obtained.

To measure PCR efficiency, serial dilutions of reverse transcribed RNA (0.1 pg to 200 ng) were amplified, and a line was obtained by plotting cycle threshold (Cp) values as a function of starting reverse transcribed RNA, the slope of which was used for efficiency calculation using the formula $E = 10^{(1/Cp - 1)}$ (16). Ribosomal 18 S RNA amplifications were used to account for variability in the initial quantities of cDNA. The relative quantitation for any given gene, expressed as fold variation over control (untreated cells), was calculated after determination of the difference between Cts of the given gene A and that of the calibrator gene B (GAPDH) in treated cells (ΔCt = CtgA − CtgB) and control cells (ΔCt = CtgA − CtgB) using the 2−ΔΔCt formula (16). GAPDH expression of a control cDNA was used as interplate calibrator. Variation over controls was determined using the above-mentioned formula among biological replicates. The effect of insulin was calculated by comparing mean expression of a control cDNA population, relative expression level between genes could be calculated based on individual Ct, provided that PCR efficiencies were close to 1. The latter were calculated according to Ref. 16 and were 1.1 ± 0.07 (mean ± S.E., n = 21), indicating an approximate doubling of DNA at each PCR cycle, as theoretically expected. The percentage of relative expression between several genes of a given family (e.g., for the three C/EBP isoforms) was calculated as follows; mean Ct of C/EBPα, C/EBPβ, and C/EBPδ in the absence of insulin were 24.9, 22.24, and 30.31, respectively. Using the 2−ΔΔCt formula, these could be expressed as two equations ($C_{EBPβ} = 6.32 × C_{EBPα}$ and $C_{EBPδ} = 268 × C_{EBPβ}$) plus another equation as $C_{EBPα} + C_{EBPβ} + C_{EBPδ} = 100$. It could thus be calculated that the percentage expression of the C/EBPα, C/EBPβ, and C/EBPδ isoforms was 13.6, 86.1, and 0.3%, respectively.

Nuclear Run-on Transcription Analysis—Differentiated 3T3-L1 cells were treated with insulin or were infected with the adenovirus encoding the DP SREBP-1c mutant. After 24 h, nuclei were prepared as previously described (17) and were incubated with [α-32P]UTP (3000 Ci/mmol) for 45 min at 32°C. Incubations were terminated by the addition of RNAse-free DNase and proteinase K, and labeled RNA was extracted by phenol/chloroform. Labeled transcripts were hybridized for 72 h with plasmid cDNA immobilized on nylon membranes. Blots were washed to high stringency, and hybridized RNA was quantified with an optical scanner (Storm 860; Amersham Biosciences).

Promoter Analysis and Transfections—A 1.4 kb promoter fragment
SREBP-1c Regulation of Gene Expression in Adipocytes

Having shown that insulin stimulated SREBP-1c in 3T3-L1 adipocytes and its potential correlation with insulin-induced gene expression profile, we assessed the ability of insulin to modulate endogenous SREBP-1c expression in these cells. In agreement with initial studies (21), we observed a stimulatory effect of insulin on SREBP-1c expression in these cells. In agreement with initial studies (21), we observed a stimulatory effect of insulin on SREBP-1c in differentiated 3T3-L1 adipocytes as described previously (20) and used for experiments, cells were infected with increasing titers of adenovirus encoding the DP or DN SREBP-1c mutants. This was confirmed by Western blot analysis with an anti-SREBP-1 antibody that showed a huge increase in SREBP-1c protein content in nuclear extracts prepared from cells infected with Ad.SREBP-1c DP (data not shown). To ensure that SREBP-mediated transcriptional activity was significantly altered in adipocytes infected with the adenoviruses encoding the DP or DN SREBP-1c mutants, we measured the steady state levels of known SREBP target genes such as FAS (5, 14, 23), SCD-1 (24), and LDL receptor (25). Fig. 1C shows that increasing titers of Ad.SREBP-1c DP mutant dose-dependently stimulated the expression of FAS, SCD-1, and LDL receptor genes (left panel). The induction of FAS, SCD-1, and LDL receptor gene expression started at 10–100 plaque-forming units/cell, and a plateau was reached after 250, the 500 plaque-forming units/cell condition being optimal. The mRNAs encoding FAS and SCD-1 were increased with higher efficiencies (up to 10-fold) than that of the LDL receptor, which was stimulated only 3-fold. This agreed well with the ability of SREBP-1c to stimulate lipogenesis in preference to cholesterol uptake in vivo (26, 27) (reviewed in Ref. 10). In reciprocal experiments, cells were infected with increasing titers of Ad. SREBP-1c DN mutant (Fig. 1C, right panel). We observed, as expected, a gradual decline in the steady state levels of FAS, SCD-1, and LDL receptor mRNAs. The observed changes in DN-expressing cells were of lesser magnitude than those in cells expressing the DP form. Since the dominant negative mutant inhibits SREBP-1c transcriptional activity by titrating endogenous SREBP-1c into inactive heterodimers (5), it is possible that Ad DN expression might not reach sufficient levels to completely inhibit endogenous SREBP-1c. Alternatively, because transcriptional activation of these genes requires in addition to SREBP other transcription factors such as NFY or Sp1 (24, 28), it remained plausible that the presence of these untrated factors or that of other co-activators allows sufficient residual transcriptional activity, thus obviating total inhibition of transcription. Taken together, all these results establish that SREBP-1c transcriptional activity can be efficiently manipulated in 3T3-L1 cells by means of adenovirus-mediated overexpression of dominant positive or negative SREBP-1c mutants.

SREBP-1c Mimics Most Insulin-induced Changes in Gene Expression 3T3-L1 Cells—The 3T3-L1 differentiated adipocyte cell system was used to compare the effects of insulin with that of SREBP-1c manipulations on the expression of various adipocyte genes. Specific primers were designed for real time fluorescent RT-PCR analyses of a panel of genes that covers a wide range of fat cell functions, i.e. lipid storage or lipolysis, transcriptional regulators of adipocyte differentiation, energy expenditure, and adipocyte-derived secreted factors (primers used are displayed in Table I). Table II shows the effects of
FIG. 1. A, endogenous expression of SREBP’s mRNAs in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 cells (day 10 postconfluence) were shifted to a serum-free medium containing 5% free fatty acid bovine serum albumin, 25 mM glucose in the absence or presence of insulin for 48 h. Then the steady state mRNA levels of three SREBP isoforms were measured using real-time RT-PCR as described under "Materials and Methods." The primers used to distinguish SREBP-1c, SREBP-1a, and SREBP-2 mRNA are shown in Table I. B, efficiency of SREBP transgene expression in 3T3-L1 adipocytes. Fully differentiated cells were infected with either Ad.SREBP-1c DP (open symbols) or Ad.SREBP-1c DN (black symbols) at various MOI from 0 to 500. After 24 h, RNA was prepared, and quantitative RT-PCRs were performed with primers designed to specifically target endogenous SREBP-1c expression (circles) and endogenous SREBP-1a expression (squares) or total SREBP-1 (triangles). After normalization to 18 S mRNA, values were expressed relative to that measured in control noninfected cells. Transduction efficiency was also evaluated visually.
insulin and that of overexpressed SREBP-1c DP and DN mutant forms on steady state levels of about 50 adipocyte mRNA species. Over the 47 genes presented here, we observed that the expression of 20 was significantly affected by insulin (see Table II, first and third gene groups) confirming that insulin profoundly influenced the tone of adipose-specific gene expression. On the other hand, a total of 27 genes were found to be modulated by SREBP-1c mutants (see Table II; first through third gene groups). Importantly, among the 20 insulin-regulated transcripts presented in Table II, all but one (Akt/PKB) were sensitive to SREBP mutant overexpression. Noticeably, SREBP-1c, which is induced by insulin (Fig. 1A), is not subjected to autoregulation (Fig. 1B). Eight genes remained (see Table II, second gene group) that were sensitive to SREBP-1c but unaffected by insulin. We suppose that such a pattern can be explained by interactions of mutants with other transcription factors of the helix-loop-helix family or cofactors that might be important for basal expression of these genes. Collectively, these data indicate that most insulin-regulated genes in adipocytes can also be modulated by SREBP-1c and establish that SREBP-1c is an important mediator of insulin action in adipose tissue.

**Identification of Novel Transcriptional SREBP-1c Target Genes**—Among the 19 genes regulated by insulin and either dominant positive or dominant negative SREBP mutants, only 10 genes exhibited coordinate increased expression with both insulin treatment and overexpression of the SREBP-1c DP form and a reciprocal decrease in cells overexpressing the SREBP-1c DN mutant (Table II, first gene group). Because of such a coordinately regulated pattern of expression, we considered these genes as being strong candidates for insulin regulation through SREBP-1c (Fig. 2). These genes encode FAS, LDL receptor, HMG-CoA reductase, high density lipoprotein receptor SR-BI, SCD-1, GAPDH, GLUT1, PAI-1, and the /H9252 and /H9254 isoforms of C/EBP. The transcriptional control of FAS (29), GAPDH (30), GLUT1 (31), SCD-1 (32), PAI-1 (33), and C/EBP /H9252 and -/H9254 (34) by insulin has been demonstrated in adipocytes or in other cell types for the LDL receptor (35) and SR-BI (36).
SREBP-1c Regulation of Gene Expression in Adipocytes

Table II
Effect of insulin, SREBP-1c DP, and SREBP-1c DN mutants on gene expression in 3T3-L1 adipocytes

Mature 3T3-L1 adipocytes were treated for 48 h as described under "Materials and Methods" with four different conditions: with insulin and Ad.Null, without insulin and Ad.Null, with Ad.SREBP-1c DN and insulin, and with Ad.SREBP-1c DP and no insulin. Steady-state levels of mRNA of a panel of about 50 genes were analyzed by quantitative real time RT-PCR. The effect of insulin was assessed by comparing C_\text{t} values obtained with insulin and without insulin; the effect of the overexpression of the dominant positive SREBP-1c mutant by comparing values obtained in Ad.SREBP-1c DP with Ad.Null insulin conditions; and the effect of the overexpression of the dominant negative SREBP-1c mutant by comparing values obtained in Ad.SREBP-1c DN with Ad.Null insulin conditions. Results are expressed as \text{-fold} variation over respective controls. \text{-fold} variations inferior to 0 were expressed as negative numbers (e.g., a \text{-fold} variation of 0.50 is expressed as \text{−2.00}). For more details, see "Materials and Methods." Results presented are means ± S.E. of 4–6 experiments. Statistical significance: *, \(p<0.05\); **, \(p<0.01\); ***, \(p<0.001\).

| Genes with coordinate changes in expression with insulin and SREBPs | Effect of Insulin | Effect of DP | Effect of DN |
|---------------------------------------------------------------|------------------|-------------|-------------|
| C/EBP\(\beta\)                                              | 1.83*            | 3.41***     | −2.17***    |
| C/EBP\(\delta\)                                             | 5.04**           | 2.55*       | −1.58       |
| FAS                                                          | 5.71***          | 5.90***     | −2.94***    |
| GAPDH                                                        | 9.95***          | 3.48***     | −2.34*      |
| GLUT1                                                        | 5.24*            | 4.31*       | −2.21       |
| HMG-CoA reductase                                            | 3.81**           | 3.86**      | −2.97*      |
| LDL-R                                                        | 7.26***          | 2.53*       | −2.79**     |
| PAI-1                                                        | 8.62***          | 4.67***     | −2.10       |
| SCD-1                                                       | 2.32*            | 2.81*       | −1.46       |
| SR-BI                                                       | 2.19**           | 2.73***     | −2.19       |

| Genes unaffected by insulin but regulated by SREBP mutants |
|----------------------------------------------------------|
| ABC1                                                      | −1.14            | −2.86*      | −1.47       |
| C/EBP\(\beta\)                                             | 1.24             | 2.94***     | 1.11*       |
| Caveolin-1                                                 | 1.13             | 3.23***     | −2.43*      |
| HSL                                                        | −1.95            | −4.17*      | −2.11       |
| LPL                                                        | −1.05            | −2.56***    | −1.69       |
| LXR                                                        | 1.14             | −2.13**     | −1.30       |
| PI3K                                                       | −1.03            | 1.22        | 3.04*       |
| PLTP                                                       | 1.03             | 3.28***     | 1.46        |

| Genes regulated by insulin but not coordinately by SREBPs |
|----------------------------------------------------------|
| Akt/PKB                                                   | −2.44**          | 1.08        | 1.31        |
| \(\beta\)-AR                                               | −2.00            | −2.70*      | −2.70*      |
| GAPDH                                                      | 3.54***          | 3.70***     | −3.74       |
| GLUT4                                                      | 4.71***          | 1.05        | −2.54*      |
| Id1                                                       | 1.73*            | −1.41       | −4.59***    |
| Id2                                                       | 3.04***          | 4.20***     | 1.23        |
| Leptin                                                     | 1.86*            | −6.25***    | −2.51*      |
| FERRIP                                                    | 2.34             | 2.94*       | −2.77*      |
| PPAR\(\alpha\)                                             | 2.04*            | 1.70        | −2.67*      |
| Resistin                                                   | 3.20***          | 1.24        | 4.92*       |

| Genes with unchanged expression with insulin or SREBPs |
|----------------------------------------------------------|
| \(\beta\)-actin                                           | 1.69             | −1.29       | −1.68       |
| \(\alpha\)-2AR                                             | 1.88             | −1.23       | −1.04       |
| Angiotensinogen                                            | 2.30             | 1.46        | 1.59        |
| aP2                                                        | 1.39             | −1.65       | −1.88       |
| AT1                                                       | −1.79            | 1.16        | 1.07        |
| \(\beta\) 1-AR                                              | −1.03            | 1.24        | 1.33        |
| \(\beta\) 2-AR                                              | −1.24            | 1.49        | 1.65        |
| BNP                                                        | −1.02            | 1.10        | 1.56        |
| Cardiotrophin-1                                            | −1.12            | −1.41       | −1.32       |
| Caveolin-2                                                 | −1.69            | −1.85       | −1.44       |
| CD36                                                       | −1.18            | −2.12       | −1.59       |
| Hexokinase II                                              | 1.04             | 1.21        | 1.46        |
| Id3                                                       | 1.78             | 1.17        | 1.09        |
| Insulin receptor                                           | 1.27             | −1.13       | 2.06        |
| RPL19                                                      | −2.83            | −1.04       | 1.77        |
| TNF \(\alpha\)                                             | −1.12            | −1.35       | 1.03        |
| UCP2                                                       | −1.12            | 1.87        | 1.56        |
| UCP3                                                       | 1.44             | 1.37        | 1.23        |
| VLDL-R                                                     | −1.48            | −1.48       | 1.15        |

Functional insulin-responsive sequences have also been described in the promoter region of GAPDH (37) and GLUT1 (38). Five of the insulin-regulated genes in Fig. 2, namely FAS (5, 14, 22), SCD-1 (24), LDL receptor (25), HMG-CoA reductase (39), and SR-BI (40), have been previously characterized as direct SREBP targets, underlying the importance of SREBP-1c for insulin effects on gene expression. It is worth mentioning, however, that insulin-regulated expression of SR-BI and LDL receptor was demonstrated in nonadipocyte cell types, thus raising the question of its physiological significance in fat cells. In particular, whether insulin notably influences the metabolism of cholesterol-rich lipoproteins in adipose tissue is an issue that has to be clarified. Interestingly, the present data point out that five other adipocyte genes were regulated in parallel by insulin and SREBP-1c (i.e., C/EBP\(\beta\), C/EBP\(\delta\), GLUT1, PAI-1, and GAPDH), suggesting that they might be new SREBP-1c targets. To assess the existence of a transcriptional control by SREBP-1c, we performed run-on transcription experiments on differentiated fat cells infected with the Ad.SREBP-1c DP or treated by insulin. Results in Table III clearly establish a positive effect of SREBP-1c on the transcription of FAS, a typical SREBP-1c target gene, but also on the C/EBP\(\beta\), C/EBP\(\delta\), and PAI-1 genes. It is noteworthy that the magnitude of the stimulation of the transcription of these genes by insulin and SREBP-1c were in a very similar range. This demonstrated that C/EBP\(\beta\), C/EBP\(\delta\), and PAI-1 are transcriptionally controlled by SREBP-1c in adipocytes. By contrast, although insulin stimulated the transcription of GAPDH and GLUT1, we
could not detect any direct effect of SREBP-1c overexpression on the transcription of these two genes. Noticeably, the two insulin-responsive elements of GAPDH localized between bases −480 and −269 (37) or that reported for GLUT1 at −2.7 kb within intron 2 (38) do not match with putative SREBP binding sequences that could be found using the TransFac database (55). Thus, these results demonstrate that C/EBPβ, C/EBPδ, and PAI-1 are new transcriptional targets of SREBP-1c in the adipocytes.

**Some Insulin Genic Actions May Not Be Mediated through SREBP-1c—** Some other genes (Table II, third gene group) were differentially regulated by the dominant negative SREBP-1c mutant, a significant decrease in resistin expression was noted. This might suggest that SREBP-1c is required for sustained expression of the resistin gene in the fat cell. However, the lack of stimulation observed with the dominant positive mutant suggests that, for a limited number of genes, the effects of insulin might not be achieved through SREBP-1c only. Another example is the β2-adrenoreceptor gene, whose expression is repressed by insulin (48). Our study shows that indeed, the β2-adrenoreceptor mRNA was down-regulated in cells treated by insulin as well as in those overexpressing the DP mutant of SREBP-1c. However, the DN mutant was not able to raise β2-adrenoreceptor mRNA, possibly because it is already expressed at remarkably high levels in the 3T3-L1 cell line. Interestingly, the β2-adrenoreceptor gene possesses putative SREBP binding sites distal to the coding sequence at positions 6473 and 6588 relative to ATG. Thus, whether the β2-adrenoreceptor gene is a negative SREBP target, as reported for the microsomal triglyceride transfer protein gene (49) and phosphoenolpyruvate carboxkinase (50), remains to be firmly established. This would be relevant with an integrated role for SREBP-1c to promote overall energy storage by stimulating lipogenic enzymes and to favor antilipolysis by inhibiting lipolytic machinery.

**SREBP-1c Directly Transactivates the C/EBPβ Promoter—** A significant finding of the present study is the observation that C/EBPβ, C/EBPδ, and PAI-1 can now be considered as new SREBP-1c transcriptional targets. This statement is based on data showing that the expression of these genes is insulin-sensitive, stimulated by the forced expression of the dominant positive mutant, and down-regulated by the dominant negative.
SREBP-1c Regulation of Gene Expression in Adipocytes

Fig. 3. SREBP-1c transactivates the C/EBPβ promoter. Proliferating 3T3-L1 preadipocytes were cotransfected with a series of C/EBPβ promoter constructs and a vector encoding (pSV Sport ADD1) or not encoding (pSV Sport) a transcriptionally active form of SREBP-1c. Results are expressed as normalized luciferase activities (A) or as -fold stimulations by ADD1/SREBP-1c (B) and represent mean values ± S.E. from three independent experiments.

SREBP-1c mutant. Moreover, their transcription rates assessed by run-on experiments were stimulated in SREBP-1c-overexpressing cells. The importance of C/EBPβ and C/EBPδ has been clearly established in the context of the adipocyte differentiation program. They act at an early stage of the adipocyte conversion, as suggested by their expression pattern that peaks early after cell confluence (51). When induced by appropriate drugs (52), C/EBPβ and C/EBPδ can initiate a transcriptional cascade that culminates in the induction of PPARγ and C/EBPα, which in turn induces the expression of the mature adipocyte phenotype. In the present study of fully mature differentiated fat cells, C/EBPβ and C/EBPδ expression were supposed to have returned to basal levels. However, if one calculates the respective proportion of the mRNA encoding the three C/EBP isoforms in the differentiated fat cells, which can be done under the real time RT-PCR conditions of this study (see “Materials and Methods”), it can be found that the steady state levels of C/EBPβ were still very high in the fully mature adipocyte and most abundant among the three C/EBP isoforms. Upon insulin treatment, C/EBPβ mRNA was largely predominant, representing 82% of total C/EBP mRNA. C/EBPα and C/EBPδ then account for 17 and 1%, respectively. Similarly, in the SREBP-1c DP-overexpressing cells, C/EBPβ mRNA represented 98% of the total C/EBP transcripts. This relative abundance of the C/EBPβ mRNA among the other isoforms suggests that the increase in the C/EBPβ mRNA levels by insulin and SREBP-1c might play a significant role on the mature adipocyte gene transcription program.

To further establish the direct control of SREBP-1c on C/EBPβ transcription, we performed cotransfection experiments in which was tested the ability of an SREBP-1c-expressing vector to activate the C/EBPβ promoter controlling the expression of the luciferase reporter gene. Fig. 3 shows that in the context of the proliferating 3T3-L1 cells, in which SREBP-1c expression is virtually absent, basal C/EBPβ promoter activity is low, with luciferase expression exceeding only by 2-fold that obtained with the promoterless pGL3 construct. This fits with the known very low expression of C/EBPβ in proliferating 3T3-L1 preadipocytes. At this stage, in the absence of endogenous SREBP-1c, ectopic expression of this transcription factor was able to transactivate the 1.4-kb C/EBPβ promoter construct, with a 4-fold stimulation of luciferase reporter expression (Fig. 3A). However, transactivation by SREBP-1c could not be observed using shorter promoter constructs, indicating that the SREBP-1c-responsive region in the C/EBPβ promoter was located upstream of −441. Using the TransFac data base (53) and the TFSearch algorithm aimed at searching transcription factor-binding sites (available on the World Wide Web at http://www.rwcp.or.jp/papia/), we identified two consensus sequences for potential SREBP binding sites in the 1.4-kb promoter sequence, one located at −1124 (SRE1) relative to the transcription start site and the other at −1064 (SRE2). These sites are located within the SREBP-1c-responsive fragments identified in the cotransfected experiments, suggesting that they might be involved in SREBP-1c responsiveness of the C/EBPβ promoter. 50 bp upstream of these SRE sites, we also found a Sp1 binding sequence, the presence of which was shown to be required for efficient transcriptional activation by SREBP-1c (10). This further suggested that the SRE sequences identified here on the C/EBPβ promoter might be functional. Fig. 3B shows the results of experiments in which point mutations in these SRE sites have been introduced. The mutation of the SRE1 sequence completely abolished the ability of cotransfected SREBP-1c to transactivate the 1.4-kb C/EBPβ promoter. On the other hand, the response of the promoter was severely blunted when the SRE2 site was mutated. In addition, in a construct bearing a double mutation of both SRE1 and SRE2 sites, no transactivation by SREBP-1c could be observed. This clearly demonstrated that SREBP-1c responsiveness of the C/EBPβ promoter relies on the presence of two identified SRE sites and suggests a predominant functional effect of the upstream SRE1. To further establish the direct control of C/EBPβ promoter activity by SREBP-1c and by insulin, we addressed the question of the localization of the insulin-responsive region in C/EBPβ promoter. For such purpose, we used fully differentiated and insulin-responsive 3T3-L1 cells, and we show in Fig. 4 that the full-length 1.4-kb promoter responded to the addition of insulin in the culture medium by a 2-fold stimulation of the luciferase reporter. We found that the insulin effect was highly reproducible among experiments, but of low magnitude, not exceeding a 2-fold increment, consistent with the results of nuclear run-on experiments (Table III). Fig. 4 also shows that the effect of insulin is abolished when cells were electroporated with promoter constructs bearing point mutations in one or both of the SRE binding sites. This demonstrates that these SRE sites are
products are synthesized from the single C/EBP SRE binding sites that coincide with the insulin-responsive gene. Western blot on 40 μg of nuclear extracts prepared from adipocytes treated for 24 h in the presence of insulin or after infection with Ad.SREBP-1c. Using a commercially available antibody (Santa Cruz Biotechnology), two bands corresponding to LAP and LIP were detected and quantified by densitometric scanning. The upper panel shows total C/EBP β detected and quantified by densitometric scanning. The lower panel shows the relative proportions of LAP and LIP. Results were obtained from two independent experiments performed in duplicate. Values are means ± S.E.

required for the insulin responsiveness of the C/EBP β promoter. Altogether, these results establish that SREBP-1c is a transcriptional target of SREBP-1c and insulin. Finally, having established that the effect of insulin on C/EBP β gene expression was mediated through a direct transcriptional control of SREBP-1c at the promoter level, we examined how the effects of insulin and in particular on the maintenance of the insulin-sensitive state that characterizes the differentiated adipocyte phenotype.

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