

Sterol Regulatory Element-binding Protein-1 Is Regulated by Glucose at the Transcriptional Level*

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***In vivo* studies suggest that sterol regulatory element-binding protein (SREBP)-1 plays a key role in the up-regulation of lipogenic genes in the livers of animals that have consumed excess amounts of carbohydrates. In light of this, we sought to use an established mouse hepatocyte cell line, H2-35, to further define the mechanism by which glucose regulates nuclear SREBP-1 levels. First, we show that these cells transcribe high levels of SREBP-1c that are increased 4-fold upon differentiation from a prehepatocyte to a hepatocyte phenotype, making them an ideal cell culture model for the study of SREBP-1c induction. Second, we demonstrate that the presence of precursor and mature forms of SREBP-1 protein are positively regulated by medium glucose concentrations ranging from 5.5 to 25 mM and are also regulated by insulin, with the amount of insulin in the fetal bovine serum being sufficient for maximal stimulation of SREBP-1 expression. Third, we show that the increase in SREBP-1 protein is due to an increase in SREBP-1 mRNA. Reporter gene analysis of the SREBP-1c promoter demonstrated a glucose-dependent induction of transcription. In contrast, expression of a fixed amount of the precursor form of SREBP-1c protein showed that glucose does not influence its cleavage. Fourth, we demonstrate that the glucose induction of SREBP could not be reproduced by fructose, xylose, or galactose nor by glucose analogs 2-deoxy glucose and 3-O-methyl glucopyranose. These data provide strong evidence for the induction of SREBP-1c mRNA by glucose leading to increased mature protein in the nucleus, thus providing a potential mechanism for the up-regulation of lipogenic genes by glucose *in vivo*.**

members of the basic helix-loop-helix leucine zipper family of transcription factors (1, 2). In contrast to other members of this, SREBPs are synthesized as ~1150-amino acid precursor proteins that remain bound to the endoplasmic reticulum and the nuclear envelope in the presence of sufficient sterol concentrations. Upon sterol deprivation, the precursor protein undergoes a sequential two-step cleavage process to release the NH₂-terminal portion (3). This NH₂-terminal, mature SREBP then enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis by binding to sterol regulatory elements or to palindromic sequences called E-boxes within their promoter regions (4, 5). Genes involved in cholesterol synthesis and regulated by SREBPs include the low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, squalene synthase, and SREBP-2 (6–8). Fatty acid synthetic genes such as acetyl-CoA carboxylase, fatty acid synthase (FAS) (9), stearoyl-CoA desaturase-1 and -2 (SCD) (10), ATP citrate lyase (ACL) (11), S14 (12), and glycerol-3-phosphate acyltransferase (13) are also directly activated by SREBPs.

Currently, there are three forms of SREBP that have been characterized. SREBP-1a and -1c are derived from a single gene through the use of alternate promoters that give rise to different first exons (2), and SREBP-1a is the more common isoform found in cultured cells and is a stronger activator of transcription than SREBP-1c because of a longer transactivating domain (14, 15). Transgenic mouse studies have shown that SREBP-1 plays a more active role in regulating the transcription of genes involved in fatty acid synthesis than those involved in cholesterol synthesis (15). SREBP-2 is derived from a different gene and is known to be actively involved in the transcription of cholesterologenic enzymes. It has been shown that all cultured cells analyzed to date exclusively express SREBP-2 and the -1a isoform of SREBP-1, whereas most organs, including the liver, express predominantly SREBP-2 and the -1c isoform of SREBP-1 (14).

It is known that both glucose and insulin are required for the production of fatty acids via the induction of lipogenic enzymes. The precise roles of glucose and insulin in this action are not known; however, it has been shown that glucose must be me-

Sterol regulatory element-binding proteins (SREBPs)¹ are

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¹ The abbreviations used are: SREBP, sterol regulatory element-binding protein; Bt₂cAMP, dibutyryl cAMP; azaserine, O-diazoacetyl-L-

serine; FAS, fatty acid synthase; ATP citrate lyase, ACL; stearoyl CoA desaturase, SCD; GFAT, glutamine:fructose-6-phosphate amidotransferase; PI, phosphoinositide; ALLN, N-acetyl-leucinal-leucinal-nor-leucinal calpain inhibitor; DLS, delipidated serum; DMEM, Dulbecco's minimally modified medium; OH, hydroxycholesterol; SCAP, SREBP cleavage-activating protein; FBS, fetal bovine serum; HG, high glucose; LG, low glucose; BSA, bovine serum albumin; HSV, herpes simplex virus.

tabolized for this effect and that insulin is only permissive to glucose action (16, 17). In support of a role for SREBP-1 in the induction of lipogenic genes by insulin, several different groups have shown through various lines of evidence that SREBP-1c is up-regulated by insulin *in vivo* and in primary hepatocyte cultures and that the induction of lipogenic enzymes corresponds to the increased concentration of SREBP-1c within a relatively short time period (approximately 6 h) (18–22). With respect to the influence of glucose on SREBP-1 expression, it has been shown that glucose does not induce SREBP-1 expression after a 6-h incubation (22); however, a later study showed that two lipogenic enzymes, FAS and S14, can be induced by glucose even in the absence of insulin after a 16-h incubation (19). Therefore, it remains possible that glucose can increase SREBP-1 expression, leading to the induction of lipogenic genes, and that this occurs after a longer period of time than the induction caused by addition of insulin.

Lipogenic enzymes, including FAS and acetyl-CoA carboxylase, are a group of genes involved in energy storage through fatty acid and triglyceride synthesis (23, 24). Excess amounts of carbohydrates taken up by cells are converted to triglycerides through these enzymes in lipogenic organs such as liver and adipose tissue. The lipogenic enzymes are coordinately regulated at the transcriptional level during different metabolic states (23, 24). Recent *in vivo* studies demonstrated that SREBP-1 plays a crucial role in the dietary regulation of most hepatic lipogenic genes. These include studies of the effects of the absence or over-expression of SREBP-1 on hepatic lipogenic gene expression (25–27), as well as physiological changes of SREBP-1 protein in normal mice after dietary manipulation such as placement on high carbohydrate diets, polyunsaturated fatty acid-enriched diets, and fasting-refeeding regimens (28–32). The fuel metabolism in these nutritionally challenged mice involves a time-dependent, multi-organ, complex milieu of metabolites and hormones. To dissect the molecular mechanism by which nutritional change regulates those metabolic genes, it is important to establish an *in vitro* cultured cell system to model these *in vivo* conditions.

The mouse hepatic cell line H2-35 was originally developed to induce liver-specific gene transcription in a temperature-sensitive manner by transformation with a temperature-sensitive strain of SV40 (33). Upon inducing cell differentiation by increasing the incubation temperature from 33 to 39 °C, mRNA levels for hepatic genes such as albumin, SCD-1, and hepatocyte nuclear factor 3- α were found to be increased by as much as 100-fold (33–35). Interestingly, SCD levels were also found to be regulated by both insulin and glucose in this cell line (34).

In the current study, these H2-35 cells were used as an *in vitro* model of differentiated hepatocytes to analyze the glucose induction of SREBP-1 expression. H2-35 cells were initially characterized to compare expression levels of SREBP-1a and -1c. In contrast to other hepatic cell lines, H2-35 cells expressed significant amounts of SREBP-1c. It was found that both SREBP-1a and SREBP-1c are up-regulated in a dose-dependent manner by the presence of excess glucose in the medium, with the -1c isoform being more strongly up-regulated than the -1a isoform. The induction is primarily at the level of transcription and results in an increase of the membrane and nuclear forms of SREBP-1. This glucose-induced up-regulation of SREBP-1 in H2-35 cells cannot be achieved by other monosaccharides or glucose analogs but can be inhibited by both azaserine, a glutamine:fructose-6-phosphate (GFAT) inhibitor, and the PI 3-kinase inhibitor, Ly294002.

EXPERIMENTAL PROCEDURES

Materials—Calpain inhibitor (ALLN) and Ly294002 were purchased from Calbiochem (La Jolla, CA). BSA, protease inhibitors (phenylmeth-

ylsulfonyl fluoride, pepstatin A, leupeptin, and aprotinin), L-glucose, fructose, 2-deoxy glucose, 3-O-methyl glucopyranose, mannitol, galactose, xylose, dibutyl cAMP (Bt₂cAMP), forskolin, and azaserine were purchased from Sigma. Fetal bovine serum (FBS), low glucose (5.5 mM, LG), and high glucose (25 mM, HG) media were purchased from Life Technologies, Inc. Both rabbit and mouse secondary antibodies for immunoblotting were purchased from Amersham Pharmacia Biotech. Delipidated serum (DLS) was prepared from fetal calf serum as described previously (36).

Preparation of Nuclear Extracts—Nuclear extracts were performed as described (37). In brief, cells were regularly passaged in high glucose Dulbecco's minimally modified medium (DMEM) with dexamethasone at 33 °C. 24 h prior to treatment, the cells were split into new dishes and placed at 39 °C to initiate differentiation. Cells were washed with prewarmed phosphate-buffered saline and then placed in treatment medium for 3–24 h (all experiments were performed after a 24-h incubation with the exception of the time course experiments). 2 h prior to collection, ALLN (1.25 μ g/ml) was added to the cells. After collection, cells were washed in phosphate-buffered saline containing ALLN and resuspended in buffer A (10 mM HEPES, pH 7.6, 1 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA). Pellets were passed through a 22 gauge needle 10 times and then briefly centrifuged. The pellet, containing the nuclei, was resuspended in buffer C (20 mM HEPES, pH 7.6, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA) rotated at 4 °C and then prepared for electrophoresis by addition of SDS loading buffer and boiling. The supernatant was centrifuged at 55,000 rpm, and the remaining membrane-containing pellets were resuspended in a buffer containing 50 mM Tris HCl, 2 mM CaCl₂, 80 mM NaCl, 1% Triton X-100. All buffers used contained a mixture of protease inhibitors containing phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, and aprotinin. A small portion of each sample was removed and analyzed for protein content by a BCA reaction (Pierce). Samples (50 μ g of protein) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to Hybond ECL membranes (Amersham Pharmacia Biotech).

Immunoblot Assays—Prior to immunoblotting, membranes were stained with Ponceau S stain (Sigma) to verify equal loading of proteins. Monoclonal antibody for mouse SREBP-1, 2A4, was prepared from a hybrid cell line purchased from American Type Culture Collection. Polyclonal antibody specific for mouse SREBP-1c and SREBP-2 were prepared as described in Refs. 31 and 25, respectively. All other blots were probed with the monoclonal 2A4 antibody for discrimination between the 1a and 1c isoforms unless otherwise indicated. Secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or -rabbit, and detection of immunoreactive bands was performed using the ECL kit by Amersham Pharmacia Biotech.

RNA Preparation and Northern Blotting—Total RNA was prepared from the cells with TRIzol Reagent (Life Technologies, Inc.). RNA was electrophoresed through formalin-denatured gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). The cDNA probes for SREBP-1, FAS, SCD, ACL, and 36B4 were prepared as described previously (25, 27). Probes were labeled with [α -³²P]dCTP using Megaprime DNA Labeling System kit (Amersham Pharmacia Biotech). Membranes were hybridized with the radiolabeled probes in Rapid-hyb Buffer (Amersham Pharmacia Biotech) and washed in 0.1 \times SSC, 0.1% SDS at 65 °C. Membranes were exposed to Kodak XAR-5 film for 2–5 h at -80 °C.

RNase Protection Assays—RNA was isolated from H2-35 and HepG2 cells cultured in either low glucose or high glucose DMEM using the TRIzol reagent by Life Technologies, Inc. RNase protection assays were performed with the same probes and conditions as described previously (14).

Establishment of Stable Transfectants—A reporter gene containing mouse SREBP-1c promoter (a 2.6-kilobase *Bam*HI-*Avr*II fragment from the mouse SREBP-1 genomic clone) (26) was prepared by insertion into the pGL2 luciferase construct (Promega). These constructs (purified with Qiagen maxi DNA preparation kit) were individually co-transfected with a neomycin resistance-containing vector (pMAMneo, from CLONTECH) into H2-35 cells using Superfect reagent (Qiagen). Empty pGL2 vector was also co-transfected with pMAMneo to be used as controls for the luciferase assays. TK-HSV-SREBP-1c, an expression vector of human SREBP-1c precursor with an HSV-Tag at the amino terminus under the control of the thymidine kinase promoter, was constructed as described previously (37). Transfected cell clones were selected with 500 μ g/ml G418 (Life Technologies, Inc.).

Luciferase Assay—Luciferase assay reagent (Promega) was added to lysed cell samples and luciferase activity was detected using the Micro-



FIG. 1. Western blot for SREBP-1a and -1c Isoforms in H2-35 Cells. H2-35 cells were cultured for 24 h in media containing 25 mM glucose. Nuclear extracts were prepared and subject to 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and blotted with either a polyclonal antibody specific for SREBP-1c (left-hand panel) or a monoclonal antibody that recognizes SREBP-1c, as well as the -1a isoform (right-hand panel). *, nonspecific band that binds to both antibodies.

plate Luminometer LB 96V by EG&G Berthold (Germany) and analyzed with Winglow software.

RESULTS

Characterization of the H2-35 Cell Line—All cultured cells analyzed to date express almost exclusively the 1a isoform of SREBP-1, whereas most organs, including liver, predominantly express the 1c isoform (14). In the current studies, H2-35 cells, a well differentiated mouse hepatocyte cell line (33), were evaluated to determine levels of SREBP-1c expression by RNase protection assays. Interestingly, when H2-35 cells were cultured at the differentiation temperature of 39 °C in high glucose (25 mM) media, they expressed high levels of the 1c isoform as well as the 1a isoform, whereas barely detectable levels of the 1c isoform were found in HepG2 cells (data not shown). In addition, the use of a polyclonal antibody that specifically recognizes SREBP-1c and a monoclonal antibody for both the 1c and 1a isoforms demonstrated that the 1c isoform is highly expressed at the protein level as well (Fig. 1). This is then the first report of a liver cell line in which SREBP-1c is expressed at levels comparable with SREBP-1a and is therefore an appropriate cell culture model for the study of SREBP-1c regulation in the liver.

Sterol Regulation of SREBP-1 and SREBP-2 Mature Forms—To determine the pattern of sterol regulation of SREBP-1 and -2 in H2-35 cells, both H2-35 and 293 cells (nonhepatocytes) were depleted of cholesterol and/or fatty acids and assayed for SREBP levels. When cells were provided with cholesterol and 25-hydroxycholesterol (OH) (20 and 2 µg/ml, respectively), the mature form of SREBP-2 was nearly abolished in both H2-35 and 293 cells. In comparison, SREBP-1 was only moderately reduced (Fig. 2, compare lanes 1 and 2). Interestingly, SREBP-1 seemed to be regulated by sterols to a lesser extent in the H2-35 liver cell line than it was in the 293 cells. When cells were depleted of cholesterol and fatty acids by treatment with DLS, they exhibited an increase in SREBP-1 and -2 expression (Fig. 2, lane 3). The DLS induced SREBP-2 cleavage to the nucleus was completely abolished by the addition of cholesterol and 25-OH, whereas SREBP-1 cleavage was only moderately reduced (Fig. 2, lane 4). In contrast, when oleate was added to the DLS containing media, mature SREBP-1 levels were decreased, whereas SREBP-2 levels remained relatively unchanged (lane 5). These data suggest that although mature SREBP-2 can be completely controlled by cholesterol at the level of cleavage, SREBP-1 is only partially responsive to sterol regulation and is also controlled by fatty acid levels.

Glucose Regulation of Precursor and Mature Forms of SREBP-1—Because H2-35 cells are known to differentiate at 39 °C, initial studies on the glucose regulation of SREBP-1 focused on measuring the levels of precursor and mature SREBP proteins from nuclear extracts of H2-35 cells cultured in different concentrations of glucose for 24 h at either 33 or 39 °C. It was noted that when cells were cultured for 24 h in

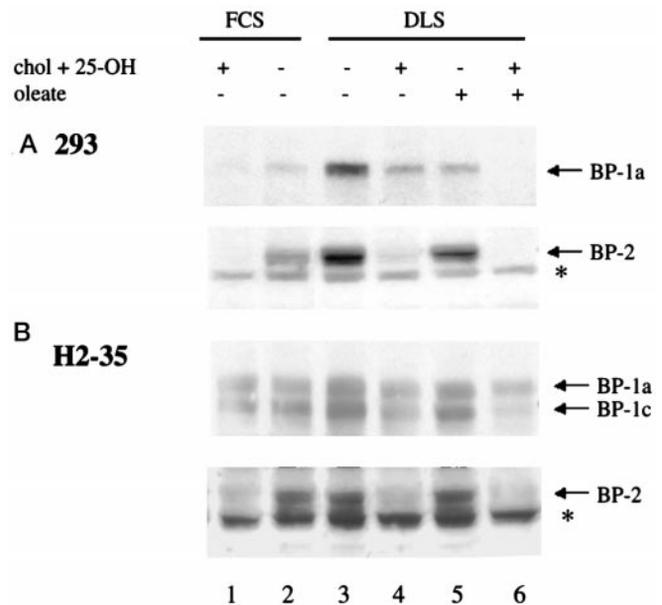


FIG. 2. Sterol regulation of SREBP-1 and SREBP-2 mature form in 293 and H2-35 cell lines. Following a 24-h incubation in the indicated culture media conditions, nuclear extracts were prepared from H2-35 and 293 cells and used for Western blot analysis as described under "Experimental Procedures." A, representative blots for SREBP-1 and SREBP-2 of nuclear extracts from 293 cells. B, blots for SREBP-1a and -1c as well as SREBP-2 of nuclear extracts from H2-35 cells. Cells in all lanes were cultured in 25 mM glucose DMEM with the following additions: lane 1, 10% FCS, cholesterol (20 µg/ml), and 25-OH (2 µg/ml); lane 2, 10% FCS; lane 3, 10% DLS; lane 4, 10% DLS with cholesterol and 25-OH; lane 5, 10% DLS plus oleate (50 µM); lane 6, 10% DLS, cholesterol, 25-OH, and oleate. The asterisks indicate an unrelated protein that cross-reacts with the respective antibodies.

2.75 mM glucose media, nuclear SREBP levels were almost undetectable, regardless of the differentiation state of the cells (Fig. 3A). Glucose levels in spent media from these cells were too low to detect. However, when media glucose concentrations were higher (5.5–25 mM), there was a notable difference in the pattern of expression of SREBP-1 as the cells converted from an undifferentiated to a differentiated phenotype. Upon differentiation at 39 °C, the levels of the precursor and mature forms of SREBP-1 were increased by glucose in a dose-dependent manner (Fig. 3A); however, when H2-35 cells were maintained at 33 °C, they expressed only moderate levels of the precursor and mature forms of SREBP-1, and these levels were not significantly influenced by media glucose concentrations. These data demonstrate that glucose responsiveness of SREBP-1 requires H2-35 cells to be in a differentiated state. The addition of mannitol to low glucose DMEM to bring the final molar concentration to 25 mM did not have any effect on SREBP-1 expression levels (data not shown), indicating that the glucose effect is not related to osmolarity. Because the antibody used for these studies is able to distinguish between the 1a and 1c isoforms, it is also of importance to note the difference in up-regulation of these two isoforms by glucose. Densitometric analysis of band intensity revealed that, although SREBP-1a (the upper band) was increased 1.9- and 2.3-fold in media containing 12.5 and 25 mM glucose compared with 5.5 mM, respectively, SREBP-1c was increased by 2.8- and 3.6-fold. These data indicate the possibility that SREBP-1c is more sensitive to glucose regulation than is SREBP-1a.

To see the effects of insulin on SREBP-1 expression, H2-35 cells were cultured in serum-free media containing BSA. Importantly, SREBP-1 was induced by HG conditions in media lacking insulin (Fig. 3B, lanes 1 and 3). The addition of 1 ng/ml of insulin to HG media increased SREBP-1 expression slightly

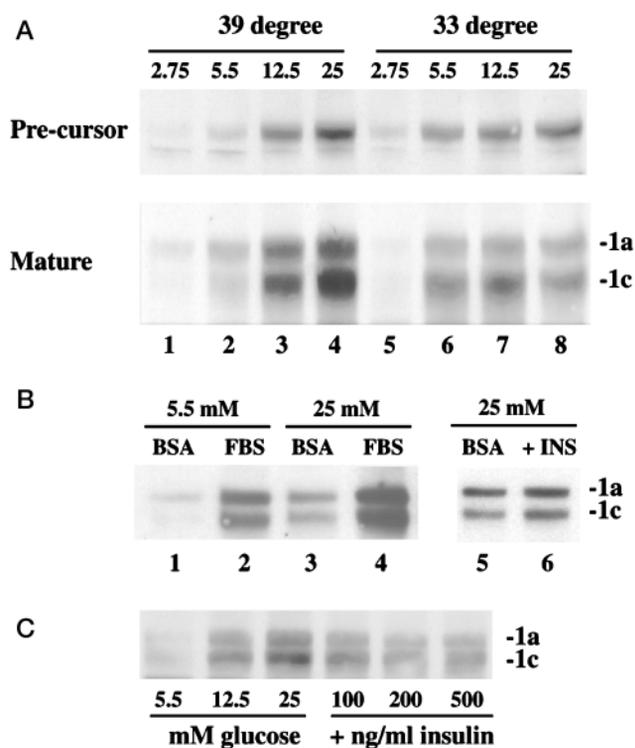


FIG. 3. Glucose stimulation of SREBP-1 precursor and mature forms at 39 °C. H2-35 cells were grown in media containing increasing concentrations of glucose for 24 h at either 33 °C or the differentiation temperature of 39 °C. Nuclear extracts and membranes were prepared and assayed for the presence of precursor and mature forms (*top* and *bottom* panels of A, respectively) of SREBP-1 by immunoblotting, according to "Experimental Procedures." Lanes 1–4, extracts from cells cultured in increasing amounts of glucose (as indicated above the lanes) at 39 °C; lanes 5–8, extracts from cells cultured at 33 °C. B, cells were grown in LG and HG media containing either FBS or BSA, as indicated. The effects of the addition of insulin can be noted when as little as 1 ng/ml was added to serum-free media. C, mature SREBP-1 from cells grown in HG FBS-containing media and treated with increasing levels of glucose and insulin (100–500 ng/ml).

(Fig. 3B, lanes 5 and 6); however, additional increases in insulin concentration did not further induce SREBP-1 expression (data not shown). Cells cultured in media containing FBS (which contains low levels of insulin) showed a substantial induction of SREBP-1 expression compared with those cultured in BSA in both LG and HG conditions (Fig. 3B). In contrast to the serum-free media results, when insulin was added to HG media containing FBS, even at very high doses (100–500 ng/ml) there was no change in expression levels of SREBP-1 (Fig. 3C). We conclude then that insulin mildly stimulates SREBP expression in differentiated H2-35 cells when added to serum-free media; however, high glucose media with FBS (and the insulin it contains) provides maximal stimulation for SREBP-1 expression. It is also important to note that a change from LG to HG, both in the complete absence of insulin (serum-free media), as well as in the presence of saturating concentrations of insulin (FBS media), resulted in the induction of SREBP-1 nuclear protein.

Time Course of SREBP-1 Regulation—To evaluate the timing of the effects of glucose on precursor and mature forms of SREBP-1, nuclear extracts from mouse liver H2-35 cells cultured in various concentrations of glucose were analyzed at 3, 6, 12, and 24 h after treatment (Fig. 4). It was found that glucose had an appreciable dose-dependent effect on both precursor and nuclear forms of SREBP starting at 12 h, with no detectable effects at earlier times. Even after 48 h, the effects of HG on SREBP-1 expression could still be detected (data not

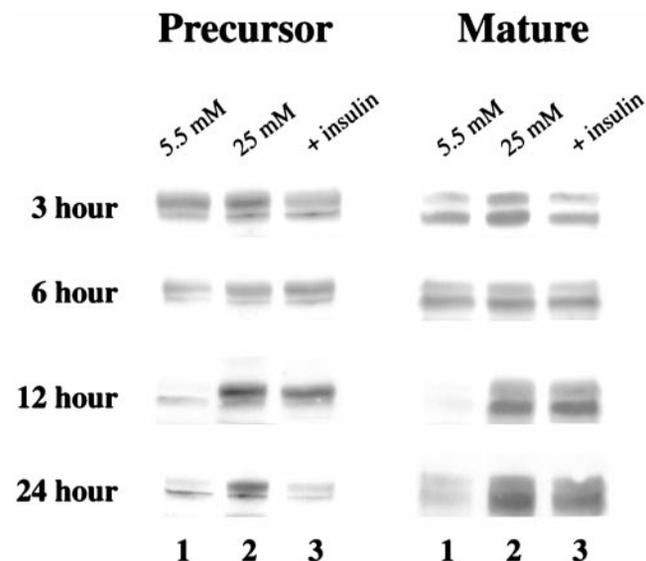


FIG. 4. Time course of glucose stimulation of SREBP-1. H2-35 cells were incubated for 3, 6, 12, or 24 h under the following conditions: lane 1, 5.5 mM glucose; lane 2, 25 mM glucose; and lane 3, 25 mM glucose + 100 ng/ml insulin. Cells were cultured in high glucose media for 24 h at 39 °C prior to beginning of time course study. Nuclear extracts and membranes were prepared and immunoblotted for SREBP-1 as described under "Experimental Procedures." The *left-hand blots* contain the precursor protein, and the *right-hand blots* contain the mature protein.

shown). The addition of insulin to the FBS-containing media did not result in any detectable changes in mature or precursor SREBP levels at any time point (Fig. 4).

SREBP mRNA Levels after Treatment by Glucose—Previous studies have focused primarily on insulin-induced transcription of SREBP-1 (18–21). The data presented in Fig. 3 clearly demonstrate that both the membrane-bound precursor form and the nuclear mature form of SREBP-1 are induced by glucose after 12 h; however, further studies were performed to determine whether the effect of glucose on SREBP-1 was at the transcriptional level. Northern blot analysis of total RNA from cells treated with increasing levels of glucose for either 4 or 24 h revealed that although there were no changes in SREBP-1 after a 4-h incubation (data not shown), there was an appreciable glucose dose-dependent increase in SREBP-1 mRNA when cells were incubated for 24 h (Fig. 5A). The mRNAs for lipogenic enzymes: FAS, ACL, and SCD were also regulated in a similar manner as SREBP-1, with the exception that FAS message was slightly increased by the addition of insulin. A probe for ribosomal protein 36B4 mRNA was used as a control for equal loading. In support of this data, RNase protection assays also showed that both the 1a and 1c isoforms were up-regulated when the glucose concentration in the media was increased from 5.5 to 25 mM (Fig. 5B), and in line with the protein data, the 1c isoform RNA was increased 4-fold, whereas the quantity of SREBP-1a RNA was only doubled.

Luciferase Assays—To further analyze the glucose effect on SREBP-1c transcription, a luciferase reporter gene was linked to the mouse SREBP-1c promoter and stably transfected with a neomycin resistance vector into H2-35 cells. Control cells were transfected with an empty luciferase vector and the neomycin vector. Glucose increased luciferase activity in a dose-dependent manner (Fig. 6), up to 3.2-fold from 5.5 to 25 mM glucose, a level similar to the SREBP Northern blot data (Fig. 5) as well as the protein data (Fig. 3). Together, these studies indicate that the majority of the glucose-regulatable SREBP expression is due to an increase in transcription.

Alternate Energy Sources—The time course of glucose-induc-

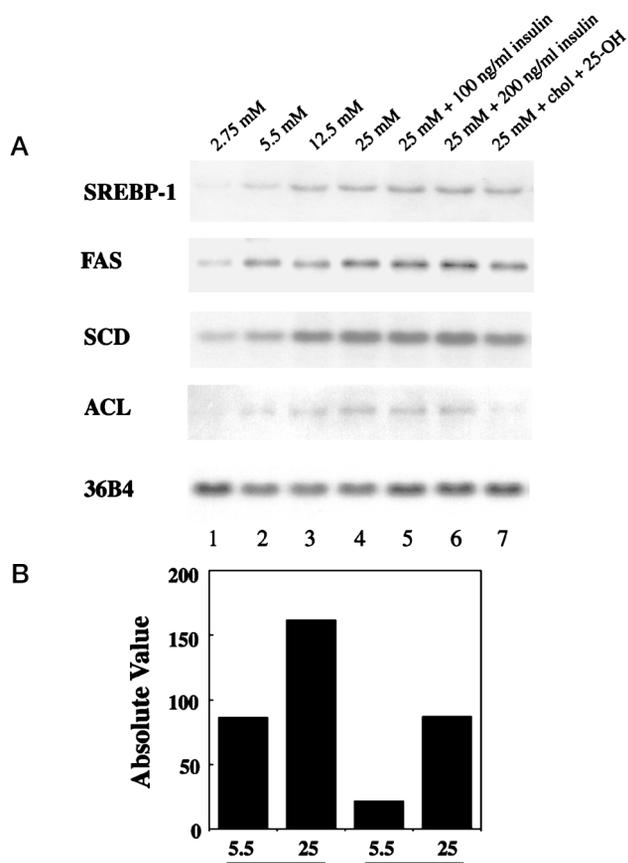


FIG. 5. SREBP-1 and FAS mRNA levels in H2-35 cells. *A*, Northern blot analysis: H2-35 cells were cultured in media containing increasing amounts of glucose. After 24 h, total RNA was extracted from cells according "Experimental Procedures." Aliquots of RNA (15 μ g) were electrophoresed through formalin-denatured agarose gels and then transferred to nylon membranes. Membranes were probed with 32 P-labeled cDNA for SREBP-1, FAS, ACL, SYN, SCD, and 36B4 (as indicated). *B*, RNase protection assay. RNase protection assays on the same RNA samples were performed to distinguish between the 1a and 1c isoforms (as indicated).

tion of SREBP-1 indicates that it may be necessary for glucose to be metabolized for its effects. Further analysis of glucose-stimulated expression of SREBP focused on this issue. Two glucose analogs, 3-*O*-methylglucose, which cannot be metabolized, and 2-deoxyglucose, which can be metabolized only as far as glucose-6-phosphate, as well as L-glucose, were unable to increase nuclear SREBP-1 levels (data not shown). Other natural monosaccharides were also used in place of glucose to determine whether they could have a similar effect on SREBP expression. Fructose, xylose, and galactose were able to up-regulate SREBP-1 only slightly compared with glucose; however, when fructose was used at a 10-fold higher concentration than glucose, comparable levels of nuclear SREBP-1 protein were detected (Fig. 7). These data demonstrate that glucose must be metabolized further than glucose-6-phosphate for SREBP-1 levels to be increased.

Metabolic Pathways Potentially Involved in SREBP-1 Regulation—To determine the role the hexosamine pathway plays in SREBP expression, H2-35 cells were treated with azaserine, a drug that irreversibly inhibits GFAT, the first enzyme in this pathway, which transfers the amide group from glutamine to fructose-6-phosphate to form glucosamine-6-phosphate. Interestingly, azaserine decreased the glucose-induced up-regulation of both precursor and mature forms of SREBP-1 in a dose-dependent manner (Fig. 8), but its effects could be detected only after 12 h of incubation (data not shown).

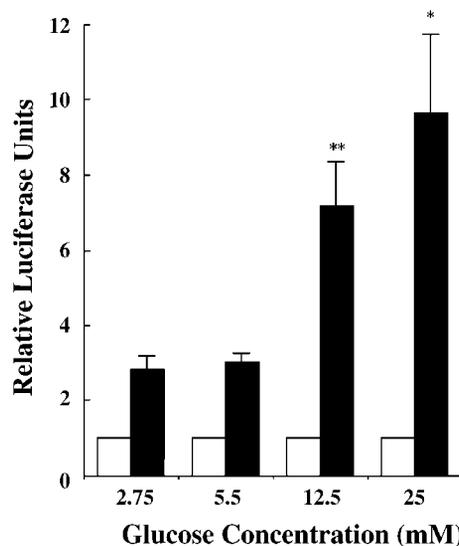


FIG. 6. Glucose effects on SREBP-1c promoter reporter construct. Stable cell lines transfected with a luciferase vector containing the SREBP-1c promoter or control empty vector were established as described under "Experimental Procedures." These stable cell lines were then grown in increasing concentrations of glucose for 24 h. Cells were lysed, and the lysate was analyzed for luciferin emission. Control bars (solid bars) are the average of three samples. The SREBP-1c luciferase promoter cells (hatched bars) are the averages of five samples from two separate experiments. Error bars represent standard error. *, $p < 0.05$, 3.2-fold increase over 5.5 mM glucose-treated cells. **, $p < 0.01$, 2.4 fold increase over 5.5 mM glucose-treated cells.

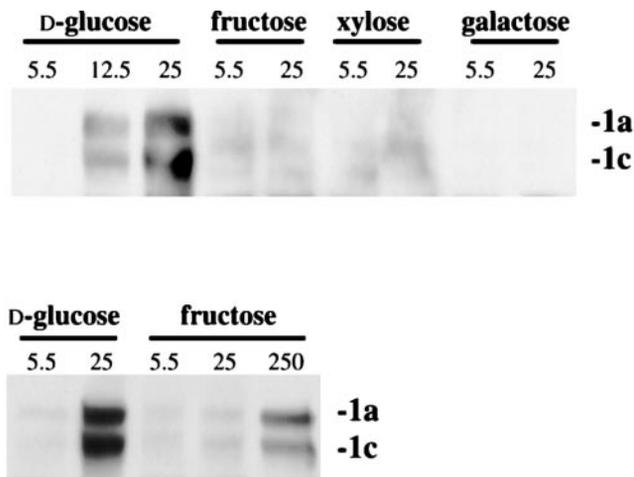


FIG. 7. SREBP nuclear protein in cells treated with alternate monosaccharide sources. H2-35 cells were cultured for 24 h in glucose-free DMEM containing 5.5, 12.5, or 25 mM glucose, or 5.5 and 25 mM of fructose, xylose, and galactose, as indicated. Nuclear extracts were prepared and SDS-PAGE was performed as described under "Experimental Procedures." The bottom panel contains the addition of a sample that was culture in 250 mM fructose (lane 3).

It has recently been shown that different signal transduction pathways are involved in the regulation of expression of metabolic genes. Of particular interest is PI 3-kinase, which has been shown to be a key switch in the insulin signaling of metabolic enzymes such as FAS, glycogen synthase, ACL, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase (reviewed in Ref. 38). To determine the contribution of the PI 3-kinase cascade to SREBP expression, H2-35 cells were treated with Ly294002, a drug that potently inhibits PI 3-kinase. Ly294002 was demonstrated to have a strong dose-dependent inhibitory effect on SREBP-1 expression at a concentration as low as 10 μ M, (data not shown) implicating a

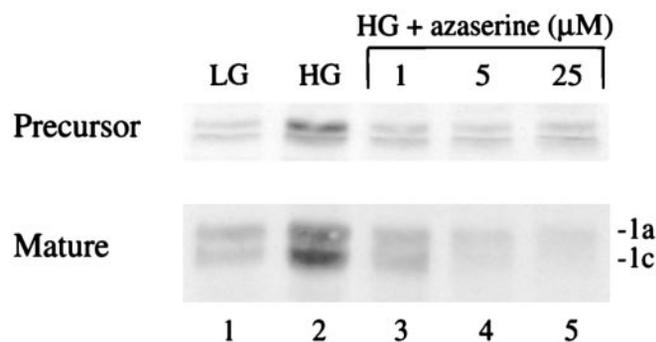


FIG. 8. **Effects of GFAT inhibition on SREBP expression.** H2-35 cells were cultured for 24 h in low glucose or high glucose DMEM (lanes 1 and 2, respectively). Lanes 3–5, nuclear protein from cells that were cultured in high glucose media with the addition of 1, 5, or 25 μM azaserine, respectively. The top panel is of the precursor form, and the bottom panel is the mature form of SREBP-1. Nuclear extracts and membranes were prepared, electrophoresed, and blotted as described under "Experimental Procedures."

potential role for the PI 3-kinase cascade in the expression of SREBP. Although the glucose induction of SREBP expression could not be detected until after 12 h of incubation, Ly294002 caused a considerable reduction in basal levels of SREBP precursor and nuclear forms as early as 3 h and was maintained for up to 24 h after addition to media (Fig. 9A).

Observations from *in vivo* experiments show that fasting, a condition that promotes gluconeogenesis through increasing plasma glucagon concentrations, completely suppresses SREBP-1 expression (32). Because glucagon exerts its effects primarily through the activation of cAMP, cAMP is likely to be involved in the fasting suppression of SREBP-1. In light of this, the effects of cAMP activation on SREBP-1 expression were examined. Forskolin, which activates adenylate cyclase leading to increased cAMP levels, and Bt_2cAMP , a cAMP analog, were added to H2-35 cells in a time course experiment. The induction of cAMP by the addition of 100 μM of each drug led to a decrease in SREBP-1 expression at 3 h after incubation; however, in contrast to the Ly294002 effects, SREBP-1 reverted to nearly base-line levels by 24 h after incubation with these drugs (Fig. 9B).

Glucose and Drug Effects on Cells Stably Transfected with HSV-tagged TK-SREBP-1c—To estimate the effect of glucose concentration on cleavage of SREBP-1c precursor into the nucleus, a construct containing an HSV-tagged SREBP-1c under the thymidine kinase promoter (37) was stably transfected into H2-35 cells. Expression of the precursor form of the transfected SREBP-1c detected by epitope (HSV) tag antibody in these cells is not regulated, allowing for the analysis of cleavage of SREBP without the added component of transcriptional variations. To estimate the sterol regulated cleavage of the tagged SREBP-1c, the cells were first cultured for 24 h with cholesterol and 25-OH, pravastatin, or oleate. It was demonstrated that SREBP-1c was cleaved to the mature form, but the addition of sterols only partially affected the cleavage of SREBP-1 in a similar manner to the endogenous protein (Fig. 10A). The membrane-bound SREBP-1c remained unchanged under these conditions (data not shown). These cells were then cultured for 24 h in media with increasing levels of glucose as well as with the addition of azaserine or Ly294002. There was very little variation in the level of precursor protein expressed between all samples (Fig. 10B). In comparison with previous experiments on endogenous SREBP-1 levels, when the amount of the precursor protein was stable, there was no change in amounts of the mature form of the protein upon increasing glucose concentrations. Therefore, there was little, if any, effect of glucose concentration on the cleavage of SREBP-1 precursor. In

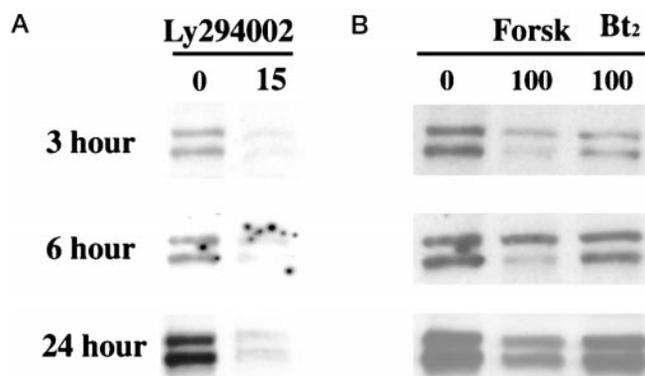


FIG. 9. **Effect of Ly294002, forskolin, and Bt_2cAMP on SREBP-1 expression.** Nuclear extracts were prepared from cells cultured for 3, 6, or 24 h in 25 mM glucose DMEM with the addition of 15 μM Ly294002, 100 μM forskolin, or 100 μM Bt_2cAMP , as indicated. Nuclear extracts were prepared and SREBP-1 Western blotting performed as described under "Experimental Procedures."

contrast, both azaserine and Ly294002 caused a reduction in the level of mature SREBP-1 without changing expression of the precursor form. These data indicate the potential action of azaserine and Ly294002 at the cleavage level for the regulation of nuclear concentrations of mature SREBP-1c.

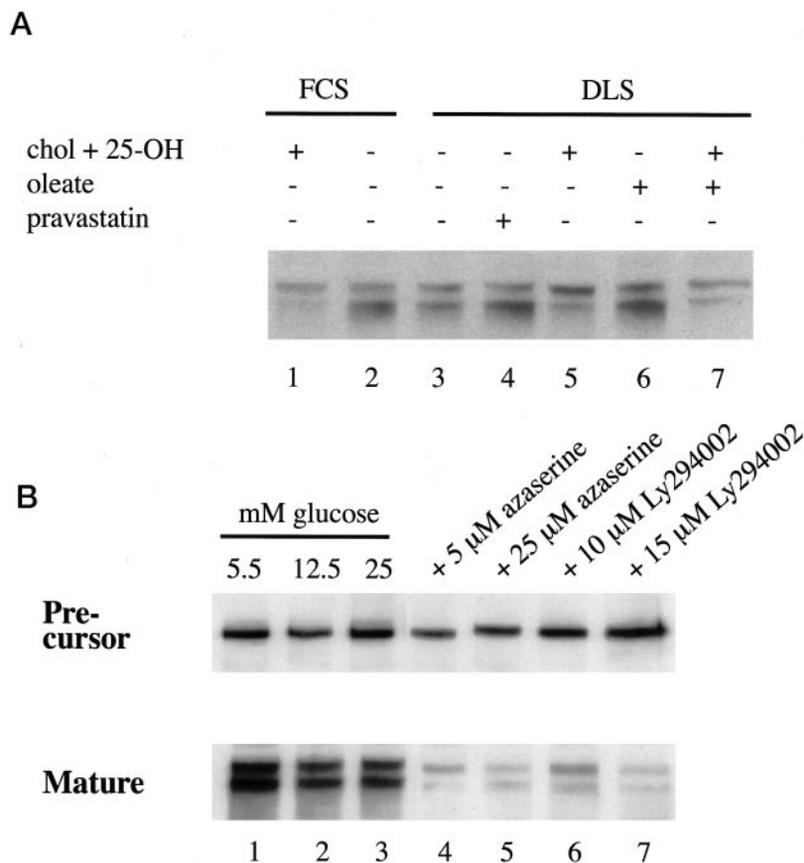
DISCUSSION

In this study, we demonstrate that both precursor and nuclear forms of SREBP-1 are up-regulated by glucose in a dose-dependent fashion. Based upon Northern blot analysis, RNase protection assay, and reporter gene assays, this up-regulation appears to be almost entirely due to an increase in SREBP-1 transcription. Time course studies demonstrated that the change in SREBP-1 concentrations occurs only after 12 h of incubation in the corresponding media, suggesting that the induction might be mediated through some metabolite of glucose and that it takes some time to have an effect.

SREBP-1 Is Not Tightly Regulated by Sterols in H2-35 Cells—It has been reported that SREBP-1a and -2 are tightly regulated by intracellular sterol concentrations at the cleavage level in cultured cells (2), as we observed in the 293 cells in the current study. Addition of cholesterol and 25-OH cholesterol, a more strongly suppressive oxysterol, abolished the cleavage of SREBP-2 almost completely, resulting in the disappearance of its mature form. These data correspond with previous data indicating that sterol regulation is the primary mechanism that dominates cleavage of SREBP-2 precursor, which, in complex with the putative cholesterol sensor, SREBP cleavage-activating protein (SCAP), translocates to the Golgi in the absence of sterols, where SREBP is cleaved by site-1 protease (1, 3). In contrast, endogenous and tagged SREBP-1 were only partially suppressed by sterols in the H2-35 liver cell line. This relatively low sensitivity to cholesterol in a hepatic cell line correlates with the observations from *in vivo* studies in mouse liver. In livers of cholesterol fed mice, the amount of nuclear SREBP-1 protein is decreased but is not completely abolished in the way that SREBP-2 is.² Together, these data indicate that there may be a difference in sensitivity to sterol suppression between cultured cells and tissues and between SREBP-1 and SREBP-2, possibly through a variance in interaction with SCAP, suggesting that SREBP-1 has the capability to enter the nucleus even in the presence of cholesterol. More resistance of hepatic SREBP-1c to sterol suppression is important for its regulation of hepatic lipogenesis. The ability of hepatocytes to remove cholesterol via the bile acid synthetic pathway may

² H. Shimano and N. Yahagi, unpublished data.

FIG. 10. Stable cell line expressing SREBP-1c linked to the TK promoter and tagged with HSV. The stable cell line TK-SREBP-1c-HSV was prepared as described under "Experimental Procedures." Cells were cultured for 24 h under the indicated conditions, nuclear extracts and membranes were prepared, and Western blotting was performed in a similar manner to all other experiments with the exception that an antibody to the HSV tag was used for recognition of the recombinant protein. **A**, cells in all lanes were cultured in HG DMEM with the following additions: *lane 1*, 10% FCS, cholesterol (20 $\mu\text{g}/\text{ml}$), and 25-OH (2 $\mu\text{g}/\text{ml}$); *lane 2*, 5% FCS; *lane 3*, 5% DLS; *lane 4*, 5% DLS with 10 μM pravastatin; *lane 5*, 5% DLS with cholesterol and 25-OH; *lane 6*, 5% DLS plus oleate (50 μM); *lane 7*, 5% DLS, cholesterol, 25-OH, and oleate. **B**, the *top blot* contains the membranes, and the *bottom blot* contains the nuclear extracts. *Lanes 1–3*, extracts from cells grown in increasing levels of glucose. *Lanes 4 and 5*, azaserine was added to the cells at 5 and 25 μM . *Lanes 6 and 7*, Ly294002 was added to the cells at 10 and 15 μM .



partially explain why they are relatively more resistant to cholesterol suppression of SREBPs than other nonhepatic cells. To date, any condition that regulates SREBP-1c expression has been found to be transcriptional (12, 19, 22, 27–29, 31, 32, 39–41). However, neither SREBP-1c nor SREBP-2 can be active in regulating the transcription of target genes, lipogenic and cholesterogenic, respectively, until their mature forms are cleaved into the nucleus. Sterol-resistant cleavage of SREBP-1c in the liver might be useful to ensure that SREBP-1c can regulate its target lipogenic genes by changing the amount of SREBP-1c precursor protein. The mechanism for the differences in sterol regulation between SREBP-2 and -1c is currently unknown; however, the relative low sequence homology between their cholesterol regulatory carboxyl-terminals might be involved (32).

Glucose Dose Dependence—The current studies clearly show that glucose is a main source and key signal for SREBP-1c induction. We compared SREBP-1 expression in cells cultured in either low (5.5 mM) or high (25 mM) glucose-containing media. These glucose concentrations are commonly used to estimate the effect of glucose in cell culture studies and also reflect physiological and diabetic glucose levels in human plasma. H2-35 cells cultured in low glucose media consistently showed low expression of SREBP-1 mRNA as well as precursor and mature forms of the protein. SREBP-1 expression from cells cultured at a range of glucose concentrations from 5.5 to 25 mM demonstrated that the glucose effect is dose-dependent and that there is no threshold level for the induction of SREBP-1 expression. According to the original report on the establishment of H2-35 cells, they can be maintained in low glucose DMEM without any impairment of growth (33). In addition, it has recently been reported that there was a partial stimulation of the S14 gene in low glucose conditions, and the authors

concluded that this glucose concentration is higher than the basal level for glucose signaling (42). Thus, the reduced level of SREBP-1 at low glucose concentrations is not due to a general poor condition of the cells, and indeed, the cells appeared healthy at the time of collection. It is likely that the glucose dose dependence is representative of *in vivo* hepatic SREBP-1c expression during fasting and refeeding. When cells were cultured in media initially containing only 2.75 mM glucose, the 24 h conditioned media was depleted of glucose (data not shown), and nuclear SREBP-1 was barely detectable, which might be comparable with fasting liver conditions. In contrast, when cells were cultured in high glucose concentrations, SREBP-1c levels increased by over 3-fold, which might be equivalent to a refed state or a situation of high carbohydrate feeding. In agreement with this, it has been shown that a high sucrose diet increases SREBP-1 mature form in mouse liver (31), and the current studies support the likelihood that the excess glucose in the diet is responsible for this effect.

The Effects of Insulin versus Glucose—The current study clearly demonstrates that insulin mildly stimulates SREBP-1 expression in H2-35 cells, as would be expected, that the FBS contains enough insulin for maximal SREBP-1 expression in high glucose media, and, finally, that supplementation of FBS-containing media with additional insulin cannot boost SREBP-1 expression levels any higher. Importantly, they also show that glucose regulates SREBP-1 expression both in the presence and in the absence of insulin, making this cell line useful for differentiating between glucose and insulin effects on SREBP-1 expression. These data also correspond with a previous study in H2-35 cells, in which it was shown that SCD, a target gene of SREBP-1, can be induced by both glucose and insulin (in serum-free media) (34), an effect that may well be due to increased SREBP-1 expression.

The glucose-dependent induction of SREBP-1 suggests that glucose can be taken up by the cells in a dose-dependent manner, which might be through an insulin-independent mechanism. As other studies have alluded to, it is likely that although glucose is necessary for SREBP-1c and therefore lipogenic gene induction, insulin is merely permissive for this induction, probably through the induction of glucokinase gene expression (as demonstrated in Ref. 19) and/or the translocation of glucose transporters. It is also possible that insulin might have some effect on SREBP-1 cleavage, possibly through another insulin signaling pathway such as activation of MAP kinase (43).

Transcription versus Cleavage—Previous reports on the insulin induction of SREBP-1, as well as our current findings on its induction by glucose, have all demonstrated the induction to be at the level of transcription (19, 21, 22, 41). In our study we also show that SREBP-1c nuclear protein is induced by glucose. These data indicate that there must also be a mechanism in place for a corresponding increase in the cleavage of SREBP-1 precursor protein; otherwise the increase in transcription of SREBP would result only in an up-regulation of the precursor, membrane-bound form of the protein. To date, the sterol-sensing SCAP and site-1 protease is the only known system for cleavage of SREBPs in physiological metabolism (44), with the exception of caspase 3 cleavage during apoptosis (45). However, our data indicate that SREBP-1 is only under partial sterol regulation in differentiated liver cells. This observation points to the probability of some nonsterol-dependent, residual cleavage activity of site-1 protease for SCAP/SREBP-1c in the liver, ensuring the regulation of lipogenic genes even in the presence of excess cholesterol. This residual activity is likely related to precursor levels of SREBP-1c, because levels of the nuclear mature form of the protein were reflective of precursor protein concentrations in both endogenous SREBP-1 studies and in the tagged SREBP-1c studies. A consequence of this would be a partial overlap of sterol and glucose regulation on lipogenesis, which is reasonable as ACL provides acetyl-CoA as a substrate for both lipogenesis and cholesterologenesis. The mechanism involved in the glucose-regulated cleavage of SREBP-1 remains unclear; however, it is clear that it is not sterol-regulated. Future studies in this area will be necessary to distinguish between the classically understood mechanism of sterol-regulated cleavage of SREBP and this new insulin/glucose regulated or residual cleavage. Another possible explanation for the nonsterol-dependent cleavage is the presence of an unknown alternate cleavage system for SREBP-1c, and although the idea is intriguing, there is no evidence for this.

Contribution of Glucose Analogs and Alternate Monosaccharides Sources—H2-35 cells take 12–24 h to achieve a maximal effect of high glucose on SREBP-1 induction. This suggests that the glucose effect is not mediated through a direct signal transduction pathway. It is likely that it is necessary for glucose to be metabolized and that it is the accumulation of one of these metabolites that is responsible for the induction of SREBP-1. The experiments with glucose analogs such as 2-deoxy glucose and 3-O-methylglucopyranose indicate that it is some metabolite following glucose-6-phosphate. The strong effect of azaserine in reducing the glucose-induction of SREBP-1 suggests the possibility that some metabolite in the hexosamine pathway might be involved in maintaining cleavage of SREBP-1 precursor. Further study is needed to analyze all of the pathways from glucose-6-phosphate (the glycolytic, glycogen synthetic, pentose, and hexosamine pathways) to determine what this metabolite might be. Our data do not support the notion that other monosaccharides are involved in the induction of SREBP-1; however, these data need to be interpreted with caution, because H2-35 cells have not been analyzed for their

ability to utilize these other sugars. In fact, a previous study demonstrated that galactose cannot be used as a primary carbon source by H2-35 cells because of a deficiency in galactose-1-phosphate uridyltransferase (46).

Metabolic Pathways Potentially Involved in SREBP-1 Regulation—Recent studies have shown that in certain circumstances, glucosamine, which enters the hexosamine pathway downstream from fructose-6 phosphate, has a greater effect than glucose on processes such as the production of transforming growth factor β 1 (47), osteopontin (48), and leptin (49). In each of these studies the GFAT inhibitor, azaserine, decreased the glucose induced production of these proteins. The results of these experiments lead to some interesting possibilities concerning the contribution of the hexosamine pathway to the effects of glucose on SREBP-1 induction. It was estimated that approximately 2–4% of glucose that is taken up by cells is shunted to the hexosamine pathway by GFAT (50). In addition to glucose, other nutrients such as free fatty acids and uridine can activate the hexosamine pathway. Therefore, it has been proposed that this pathway can serve as a general nutrient sensing mechanism by cells (Refs. 49 and 51). In light of this, it is logical that cells respond to azaserine-induced blocking of the hexosamine pathway by reducing SREBP-1c, the signal for lipogenesis.

The PI 3-kinase pathway has been shown to be involved in the induction of many different lipogenic enzymes, including that of FAS and acetyl-CoA carboxylase. In addition, PI 3-kinase appears to play a role in insulin-stimulated glucose uptake (52). We demonstrate here a clear effect of the PI 3-kinase inhibitor, Ly294002, on mature SREBP-1 induction. Because SREBP-1 has been shown to be upstream from genes such as FAS (53), the effect of PI 3-kinase inhibition of FAS may be as a secondary effect of its effect on SREBP-1. Of interest is the fact that Ly294002 reduced levels of RNA and precursor forms of SREBP-1 (seen upon collection and assay of membranes and RNA from H2-35 cells; data not shown) as well as the cleavage of the precursor form (Fig. 10) in as little as 3 h. These data provide evidence that PI 3-kinase signal transduction may be involved in the activation of SREBP-1c and that blocking of this signal can interfere with the induction signals provided by glucose.

In contrast, the inhibitory effect of cAMP generation by addition of forskolin and Bt₂cAMP on SREBP-1 expression was transient. This is most likely due to a more rapid degradation of these drugs than Ly294002; however, it may have some biological significance as to the respective roles of PI 3-kinase and cAMP in stimulating lipogenesis. More studies will be needed to determine whether the effects of these drugs in inhibiting SREBP-1 expression are due to their effects on a glucose metabolite or on insulin signaling pathways.

The current studies demonstrate that well differentiated H2-35 cells express high levels of SREBP-1c compared with other hepatic cells lines and are therefore a good model to study the regulation of SREBP-1c. Although previous studies have highlighted the importance of insulin in the induction of SREBP-1 leading to regulation of downstream lipogenic enzymes, we show that glucose also plays an important role in SREBP-1 expression. Further, the SREBP-1 induction by glucose is dose- and time-dependent and is at the level of transcription. Future studies of the SREBP-1c promoter are necessary to understand the mechanism of this regulation and the corresponding effects on lipogenesis. Other studies focusing on the glucose metabolism pathways responsible for the carbohydrate response of SREBP-1 and lipogenic genes as well as possible control of cleavage by different signal transduction pathways are also of interest.

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