Hepatic Glucokinase Is Required for the Synergistic Action of ChREBP and SREBP-1c on Glycolytic and Lipogenic Gene Expression*

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Hepatic glucokinase (GK) catalyzes the phosphorylation of glucose to glucose 6-phosphate (G6P), a step which is essential for glucose metabolism in liver as well as for the induction of glycolytic and lipogenic genes. The sterol regulatory element-binding protein-1c (SREBP-1c) has emerged as a major mediator of insulin action on hepatic gene expression, but the extent to which its transcriptional effect is caused by an increased glucose metabolism remains unclear. Through the use of hepatic GK knockout mice (hGK-KO) we have shown that the acute stimulation by glucose of L-pyruvate kinase (L-PK), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and Spot 14 genes requires GK expression. To determine whether the effect of SREBP-1c requires GK expression and subsequent glucose metabolism, a transcriptionally active form of SREBP-1c was overexpressed both in vivo and in primary cultures of control and hGK-KO hepatocytes. Our results demonstrate that the synergistic action of SREBP-1c and glucose metabolism via GK is necessary for the maximal induction of L-PK, ACC, FAS, and Spot 14 gene expression. Indeed, in hGK-KO hepatocytes overexpressing SREBP-1c, the effect of glucose on glycolytic and lipogenic genes is lost because of the impaired ability of these hepatocytes to efficiently metabolize glucose, despite a marked increase in low Km hexokinase activity. Our studies also reveal that the loss of glucose effect observed in hGK-KO hepatocytes is associated with a decreased in the carboxydrate responsive element-binding protein (ChREBP) gene expression, a transcription factor suggested to mediate glucose signaling in liver. Decreased ChREBP gene expression, achieved using small interfering RNA, results in a loss of glucose effect on endogenous glycolytic (L-PK) and lipogenic (FAS, ACC) gene expression, thereby demonstrating the direct implication of ChREBP in glucose action. Together these results support a model whereby both SREBP-1c and glucose metabolism, acting via ChREBP, are necessary for the dietary induction of glycolytic and lipogenic gene expression in liver.

1 The abbreviations used are: GK, glucokinase; HK, hexokinase; G6P, glucose 6-phosphate; X5P, xylulose 5-phosphate; SREBP-1c, sterol regulatory element-binding protein-1c; Ad-SREBP-1c, recombinant adenovirus expressing SREBP-1c; Ad-null, adenovirus vector containing no exogenous gene; L-PK, L-pyruvate kinase; FAS, fatty acid synthase; L-CPT I, liver carnitine palmitoyltransferase I; ACC, acetyl-CoA carboxylase; ChREBP, carbohydrate responsive element-binding protein; ChOE, carbohydrate response element; SRE, sterol regulatory element; RT-PCR, real-time quantitative polymerase chain reaction; HCHO, high carbohydrate diet; siRNA, small interfering RNA; pfu, plaque-forming unit; KO, knockout.

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these genes, is necessary for the transcriptional effect of SREBP-1c. In addition, the recent identification of a glucose-responsive transcription factor named ChREBP (carbohydrate-responsive element-binding protein) (20) has shed light on the possible mechanism whereby glucose affects gene transcription and further supports the hypothesis that glucose acts through a distinct, but synergetic, signaling pathway through insulin to regulate glycolytic and lipogenic gene expression in liver.

These studies were undertaken: 1) to elucidate the role of the hepatic GK in the control of the transcriptional regulation of glycolytic and lipogenic gene expression by glucose, 2) to test whether the stimulatory effect of SREBP-1c is linked to glucose metabolism, and 3) to determine the direct implication of ChREBP in mediating glucose signaling in liver. For this purpose, nutritional regulation studies were performed in hepatic-specific GK knockout mice (hGK-KO mice) (6). An adenovirus expressing a dominant positive form of SREBP-1c was used in vivo and in primary cultured hepatocytes, to determine whether glucose metabolism via GK is necessary for the maximal induction of glycolytic and lipogenic gene expression by SREBP-1c. In addition, ChREBP “knock-down” experiments using small interfering RNA (siRNA) were performed in control hepatocytes to determine the direct implication of this transcription factor in glucose signaling.

We found that hepatic GK is necessary for the activation of glycolytic and lipogenic gene expression in liver, and that the synergistic action of SREBP-1c and glucose metabolism via GK is necessary for the maximal induction of l-PK, ACC, FAS, and Spot 14 gene expression. This study also reveals that ChREBP gene expression is decreased in hepatocytes from hGK-KO mice and that ChREBP gene silencing in control hepatocytes abolishes the glucose effect on l-PK, FAS, and ACC, providing the first direct evidence (in a physiological context) that ChREBP mediates glucose signaling on both glycolytic and lipogenic gene expression in liver.

EXPERIMENTAL PROCEDURES

Animals—3-5-month-old control (gk+/+ or hGK-KO) male mice (6) bred into a C57BL/6J genetic background at least for 9 generations were used for this study. All mice were housed in colony cages with a 12-h light/dark cycle. All procedures were carried out according to the French guidelines for the care and use of experimental animals. Mice had free access to water and food (in terms of energy: 65% carbohydrate, 11% fat, 24% protein, UAR).

Nutritional Studies—For the fasting and refeeding studies, mice from each genotype were divided into two groups. One was fasted for 24 h, and the refeeding group was fasted for 24 h and then refed for 18 h with a high carbohydrate diet (in terms of energy: 72.2% carbohydrate, 1% fat, 26.8% protein). After intraperitoneal pentobarbital anesthesia (70 mg/kg body weight), livers from mice of both genotypes were rapidly sampled, frozen in liquid nitrogen, and kept at −80 °C until RNA extraction or metabolic assays.

Analytical Procedures—The concentration of plasma insulin was determined by radioimmunooassay using a rat insulin radioimmunooassay kit (Diasorin). The binding reactions were modified to perform the assay on 10 μl of plasma.

Primary Culture of Hepatocytes and Treatment with Recombinant Adenovirus—Hepatocytes were isolated from the livers of fed mice by a modification of the collagenase method (21). Briefly, livers from control and hGK-KO mice were perfused with Hank’s balanced salt solution (HBSS, KCl, 5.4 mM; KH2PO4, 0.45 mM; NaCl, 138 mM; NaHCO3, 4.2 mM; Na2HPO4, 0.34 mM; glucose, 5.5 mM; HEPES, 1 mM; EGTA, 50 mM; CaCl2, 50 mM; pH 7.4). Livers were washed at a rate of 5 ml/min using the portal vein before collagenase (0.025%) was added. Cell viability was assessed by the trypan blue exclusion test and was always higher than 95%. Hepatocytes were seeded at a density of 2×106 cells in 60-mm Petri dishes for RNA extraction or 5×106 cells (in 100-mm Petri dishes for metabolite concentration assays) in medium M199 with 10% fetal bovine serum (FBS) (Gibco, Invitrogen), supplemented with 10 μg/ml of streptomycin, 100 units/ml of penicillin, 2.4 mM of glutamine, 0.1% (w/v) bovine serum albumin, 2% (v/v) Ultroser G (Invitrogen), 100 μM dexamethasone (Solu-Decadron, Merck Sharp), and 100 μM insulin (Actrapid, Novo-Nordisk). After cell attachment (6 h), the medium was replaced by fresh M199 medium for 24 h. Hepatocytes were then incubated for 120 min at 37 °C in 1 ml of M199 medium with 1 plaque-forming unit per cell (pfu/cell) of either adenovirus null (Ad-null) or adenovirus SREBP-1c (Ad-SREBP-1c), in the presence of 5 or 25 μM glucose. Then fresh medium was added, and cells were maintained in culture for 24 h; the efficiency of the adenoviral infection always approximated 90%. The adenovirus vector containing the transcriptionally active N-terminal fragment (amino acids 1–403c) of SREBP-1c (Ad-SREBP-1c) was previously described (13). The adenovirus vector containing the promoter with no exogenous gene (Ad-null) was used as a negative control (13).

Injection of Recombinant Adenovirus—Twenty-four hours fasted control and hGK-KO mice were anesthetized with isoflurane (Belamont, France) prior to the injection through the penis vein with 10 μl of either Ad-null or Ad-SREBP-1c in a final volume of 200 μl of sterile phosphate-buffered saline. Mice were studied 18 h after the injection (between 8:00 and 10:00 a.m.). Livers were then removed and frozen immediately in liquid nitrogen. Blood samples were taken by heart puncture for both glucose and insulin measurements.

Small Interfering RNA Preparation and Transfection Protocol—21- nucleotide RNA with 3′-dTdT overlaps was synthesized using 2′-O-ACE-RNA phosphoramidites by Dharmacon Research (Lafayette, CO) in the “ready to use” option, in deprotected and desalted form. The siRNA sequence targeting mouse ChREBP (GenBank accession number NM_008070) was chosen to disrupt the PK-RAS-ERK pathway. The AA-N19 mRNA targets were 5′-UGUUGGCAAUGCUGACAUG-3′ for ChREBP siRNA and 5′-AUCGUAUCAGUAUGGUGUGG-3′ for scrambled siRNA. Primary cultures of hepatocytes were transiently transfected with siRNA duplexes using the technique described by Bousif et al. (22). Briefly, after cell attachment, the medium was replaced by fresh M199 medium for 24 h. Control hepatocytes were then transfected with 200 pmol of either ChREBP or scrambled siRNA and polyethyleneimine (PEI) in M199 medium, supplemented with 100 μM dexamethasone and 100 μM insulin, in the presence of 5 μM glucose. Transfection was accomplished by dilution of PEI and siRNA in 50 μl of NaCl (150 mM). Transfection efficiency increased the expression of the firefly luciferase vector pNLS/LacZ (Rous sarcoma virus promoter driving the nlsLacZ gene) was added simultaneously to the hepatocytes (23). After transfection (5 h), the medium was changed, and control transfected hepatocytes were cultured for 24 h in M199 medium supplemented with 100 μM dexamethasone and 100 μM insulin. Then, fresh M199 medium was added for 24 h, in the presence of 5 or 25 μM glucose, 100 μM dexamethasone, and 100 μM insulin.

Enzyme Activities—Activities were measured in cell extracts prepared from hepatocytes. Around 8×106 cells were lysed in 500 μl of homogenization buffer consisting of 50 mM triethanolamine hydrochloride (pH 7.3), 100 μM KCl, 1 mM dithiothreitol, 5% glycerol, 1 μM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin. Homogenates were collected in Eppendorf vials, incubated for 10 min on ice with PEG 25%, and then centrifuged at 10,000 × g for 15 min at 4 °C. Glucose phosphorylating activities were measured by an enzymatic method that measures the production of NADPH from NADP+ in the presence of glucose 6-phosphate dehydrogenase (6PGD) (Roche Applied Science), in the presence of either 100 μM glucose and 0.5 μM glucose, to distinguish HK from GK activity. The assay buffer contained 100 mM triethanolamine hydrochloride (pH 8), 40 mM MgCl2, 200 mM KCl, 2 mM dithiothreitol, 0.2% bovine serum albumin, 5 μM ATP, 1 μM NADP, and 30 units/ml of 6PGD. The phosphorylating capacity obtained at 0.5 μM glucose is considered the hexokinase activity, whereas the substrate utilization measured at 100 μM glucose from the activity measured at 0.5 μM glucose is considered the glucokinase activity of the extract. Glucose phosphorylating activity was taken as the increase in NADPH absorbance measured at 340 nm after 3 min at 25 °C, expressed in milliunits per mg of protein. Protein concentration was measured using the Bradford method (Bio-Rad, Labouretary) using bovine serum albumin as standard.

Measurement of Glucose 6-Phosphate and Glycogen Concentrations—G6P concentrations were determined in cell extracts prepared from hepatocytes or from liver samples by an enzymatic method (24). Briefly, after removing the culture medium, 10×106 cells were washed with 200 mg of livers were homogenized in 1 ml of 4% (v/v) ice-cold perchloric acid. After centrifugation (10 min at 3000 × g), the acid supernatant was neutralized and used for spectrophotometric determination of both G6P and glycogen concentrations. A 500-μl aliquot was used for spectrophotometric determination of G6P in 0.4× tri-
analumine hydrochloride, pH 7.6, 0.5 mM MgCl₂ containing 2 mM of NADP⁺. The increased absorbance at 340 nm after addition of 30 units/ml of G6PDH was measured. Results were expressed as nmol of G6P/10⁶ cells or as nmol of G6P/mg of liver weight.

In liver samples, glycogen from the neutralized supernatant was digested (1 h at 55 °C) with 40 units/ml of α-(1→4)-(1→6)-amyloglucosidase (Roche Applied Science). Glycogen concentration in liver samples was measured by a colorimetric method using the peridochrom glucose kit reagent (Roche Applied Science), and results were expressed as mg of glycogen/mg of liver weight. Glycogen content in cultured hepatocytes was measured by an enzymatic method (9). Briefly, G6P, from the neutralized supernatant previously used for G6P and glycogen determination, was assayed spectrophotometrically in a reaction using transferralized supernatant previously used for G6P and glycogen determination, was assayed spectrophotometrically in a reaction using transketolase (EC 2.2.1.1) (Sigma) in the presence of ribose 5-phosphate, 4 mM MgCl₂, 1 mM ATP, and 30 units/ml of G6PDH. Glycogen concentration was quantified by measuring the change in absorbance at 340 nm, 10 min after addition of 30 units/ml of hexokinase (Roche Applied Science). A glucose solution (1 g/liter) was used as standard. Results were expressed as nmol of glycogen/10⁶ cells.

**Measurement of Xylose 5-Phosphate—Xylose 5-phosphate (X5P) concentrations were determined in cell extracts prepared from cultured hepatocytes by an enzymatic method (9). Briefly, X5P, from the neutralized supernatant previously used for G6P and glycogen determination, was assayed spectrophotometrically in a reaction using transketolase (EC 2.2.1.1) (Sigma) in the presence of ribose 5-phosphate, thiamine pyrophosphate, MgCl₂, coupled to trisphosphate isomerase and α-glycerophosphate dehydrogenase (Sigma) in the presence of NADH. The decreased absorbance at 340 nm after addition of 20 units/ml of transketolase was measured. Results were expressed as nmol of X5P/10⁶ cells.

**Isolation of Total RNA and Northern Blot Analysis—**Total cellular RNAs from whole liver were extracted by using the guanidinium thiocyanate method (25), and RNA from cultured hepatocytes were extracted using the RNeasy kit (Qiagen). Northern blot analyses were performed as described previously (3). Labeling of each DNA probe with ³²P-dATP was performed by random priming (Megaprime labeling kit, Amersham). Autoradiograms of Northern blots were scanned and quantified using an image processor program (Chemi Genius). The GK probe was a 2000 bp EcoRI-EcoRI insert from pUCGK1 (26). The cPT-I probe was a EcoRI-EcoRI insert from subclone β1a (27). FAS, ACC, l-PK, SREBP-1, and Spot14 were used as controls. Probes from nuclear extracts (50 µg) and from the cytoplasmic fraction (50 µg) from livers of control and hGK-KO mice were subjected to SDS-PAGE analysis on a 10% gel. Protein concentration was determined using the Bradford method (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin served as loading controls for nuclear and cytoplasmic extracts, respectively.

**RESULTS**

**Analysis of mRNA Expression by Real-time Quantitative PCR—**Total RNA (500 ng) was reverse-transcribed for 1 h at 42 °C in a 20-µl final volume reaction containing 50 mM Tris-Cl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 250 mM random hexamers (Promega), 250 ng of oligo(dT) (Promega), 2 µM each of dNTPs, and 100 units of superscript II reverse transcriptase (Invitrogen). Real-time quantitative PCR analysis was performed starting with 6.25 ng of reverse-transcribed total RNA, in a final volume of 10-µl PCR reaction, with 0.5 µM of each primer (Invitrogen), 2 mM MgCl₂, using 1× light cycle DNA Master SYBR Green I mix in a light cycle instrument (Roche Applied Science). Samples were incubated in the light cycle apparatus for an initial denaturation at 95 °C for 10 min, followed by 40 cycles. Each cycle consisted of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The specific primers used are described in Table I. SYBR Green I fluorescence emission was determined after each cycle. The relative amounts of the different mRNAs were quantified by using the second derivative maximum method of the light-cycle software. Cyclophilin was used as an invariant control, and the relative quantification for a given gene was corrected to the cyclophilin mRNA values. Amplification of specific transcripts was confirmed by melting curves profiles generated at the end of each run. PCR specificity and product length were further checked by agarose gel electrophoresis and ethidium bromide staining.

**Preparation of Nuclear Extracts and Immunoblot Analysis—**Nuclear and cytoplasmic extracts were prepared from livers of control and hGK-KO mice using the NE-PER nuclear and cytoplasmic extraction kit (Pierce), according to the manufacturer’s instructions. Proteins from nuclear extracts (50 µg) and from the cytoplasmic fraction (50 µg) from livers of control and hGK-KO mice were subjected to SDS-PAGE analysis on a 10% gel. Protein concentration was determined using the Bradford method (Bio-Rad) using bovine serum albumin as a standard. Proteins were electrotransferred onto Hybond C nitrocellulose membrane (Amersham Biosciences). SREBP-1 was detected with a mouse monoclonal antibody (SREBP-1 Ab-1, dilution 1:1000, NeoMarkers, Interchim) raised against amino acids 301–407 of human SREBP-1, for 4 h. Detection of signals was performed using the ECL Western blot detection kit (Pierce) with anti-mouse horseradish peroxidase-conjugated IgG (dilution 1:10,000, Pierce) as second antibody. Monoclonal mouse β-actin (Sigma, clone AC74, dilution 1:1000) and polyclonal rabbit Lamin A/C (Cell Signaling Technology, dilution 1:1000) antibody were used as loading controls to normalize the signal obtained respectively for membrane and nuclear SREBP-1 protein.

**Staining Techniques—**To detect the presence of lipids droplets, hepatocytes transfected with either SREBP-1 or scrambled siRNA were fixed at the end of the culture period with 6% formaldehyde in phosphate-buffered saline and stained with oil red O (28).

**Statistical Analyses—**Results are reported as mean ± S.E. The comparison of different groups was carried out using unpaired Student’s t test. Differences were considered statistically significant at p < 0.05.

**RESULTS**

**Glycolytic and Lipogenic Gene Expression Is Not Induced by High Carbohydrate Refeeding in Liver of hGK-KO Mice—**

**TABLE I**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin</td>
<td>Sense, Antisense</td>
<td>5'-AGGCTCTGCGGAGCTCC-3'</td>
<td>125</td>
</tr>
<tr>
<td>Hexokinase II</td>
<td>Sense, Antisense</td>
<td>5'-TGGCACTTCTGAGCCAA-3'</td>
<td>202</td>
</tr>
<tr>
<td>Hexokinase I</td>
<td>Sense, Antisense</td>
<td>5'-GATGCTGCTTATGCGAC-3'</td>
<td>155</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Sense, Antisense</td>
<td>5'-CTGGGAGCTCAAAGAG-3'</td>
<td>155</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>Sense, Antisense</td>
<td>5'-GAAGCCACCTTAGATGCCC-3'</td>
<td>143</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sense, Antisense</td>
<td>5'-GGCTCTGAGGTGCAAGGACC-3'</td>
<td>122</td>
</tr>
<tr>
<td>FAS</td>
<td>Sense, Antisense</td>
<td>5'-AAGGGTCCTCCAGCTAG-3'</td>
<td>137</td>
</tr>
<tr>
<td>l-PK</td>
<td>Sense, Antisense</td>
<td>5'-ATTGGGAGGTGAGCGAGGACC-3'</td>
<td>97</td>
</tr>
<tr>
<td>ACC</td>
<td>Sense, Antisense</td>
<td>5'-ACCTCTGCTGGGTAGGACC-3'</td>
<td>97</td>
</tr>
</tbody>
</table>
determine whether the lack of hepatic GK impairs the acute regulation of glycolytic and lipogenic genes by a high carbohydrate (HCHO) diet, we first performed fasting and HCHO refeeding studies in control and hGK-KO mice. In livers of control mice, GK, SREBP-1, L-PK, ACC, FAS, and Spot 14 mRNA concentrations were low after a 24-h fast but were markedly increased upon HCHO refeeding (Fig. 1A). Similarly, mRNA levels for SREBP-1, L-PK, ACC, FAS, and Spot 14 were low in liver of 24-h fasted hGK-KO mice (Fig. 1A). However, in contrast to control mice, HCHO refeeding did not induce L-PK, ACC, FAS, and Spot 14 gene expression in liver of hGK-KO mice, despite a normal induction in SREBP-1 gene expression (Fig. 1A). SREBP-1c is synthesized as a precursor form anchored within the endoplasmic reticulum, and since its activation requires a proteolytic cleavage allowing the transcriptionally mature active part of SREBP-1c to be translocated into the nucleus (29), we examined precursor and nuclear SREBP-1c protein levels in liver of control and hGK-KO mice previously used for mRNA analysis (Fig. 1B). We used an antibody that recognizes both SREBP-1 isoforms (-1a and -1c), but since liver predominantly expresses SREBP-1c (30), we assumed that the signal obtained in liver of control and hGK-KO mice was mainly due to the expression of the isoform 1c. Precursor levels of SREBP-1c were low in both control and hGK-KO mice after a 24-h fast, and increased to similar levels upon HCHO refeeding (Fig. 1B). Mature SREBP-1c, which was not detectable in nuclear fractions from 24-h fasted control and hGK-KO mice, was also induced to comparable levels in nuclear extracts from 18-h refed control and hGK-KO mice (Fig. 1B). Thus, the lack of induction of glycolytic and lipogenic genes in liver of hGK-KO mice was not due to an altered maturation of SREBP-1c. Plasma insulin concentrations in both groups of mice were similar either in the fasted (342 microunits/ml ± 50.6 for control versus 285 microunits/ml ± 30.2 for hGK-KO mice) or in the refed state (450 microunits/ml ± 122.5 for control versus 631 microunits/ml ± 231.8 for hGK-KO mice). Moreover, in order to check the nutritional status of the mice we also measured mRNA levels of the 1-CPT I (Fig. 1A), a gene known to be induced during starvation and repressed by HCHO refeeding (27, 31). In livers of control and hGK-KO mice, 1-CPT I mRNA concentrations were markedly increased after a 24-h fast and were similarly repressed upon HCHO refeeding (Fig. 1A), demonstrating that both groups of mice were correctly refed.

These results demonstrate (i) the absence of GK in the liver of hGK-KO mice prevents the induction of glycolytic and lipogenic gene expression after HCHO refeeding and (ii) that glucose phosphorylation through GK is not necessary for SREBP-1c gene expression, maturation, and, for its nuclear abundance.

Adenovirus-mediated Overexpression of SREBP-1c in Liver of Control and hGK-KO Mice—To determine whether SREBP-1c, when it is overexpressed at higher than physiological levels, is able to induce glycolytic and lipogenic gene expression in the absence of GK, we overexpressed the transcriptionally active form of SREBP-1c (Ad-SREBP-1c) in livers of 24-h fasted control and hGK-KO mice using an adenovirus strategy. As previously described, the injection of SREBP-1c adenovirus (109 pfu) results in a marked expression of the transgene in liver (Fig. 2A) (32), whereas its expression remains undetectable in other tissues (data not shown). While glycolytic and lipogenic gene expression remained low in livers of 42-h fasted control and hGK-KO mice (Fig. 2A), overexpression of SREBP-1c for 18 h markedly stimulated the expression of GK and FAS in the liver of fasted control mice. Surprisingly, it also led to a marked stimulation of FAS expression to comparable levels than com-

**Fig. 1.** Fasting and high carbohydrate refeeding in control and hGK-KO mice. A, Northern blot analysis of glycolytic and lipogenic genes from livers of fasted and refed control and hGK-KO mice. Control and hGK-KO mice were divided into two groups. One group was fasted for 24 h, and the refed group was fasted for 24 h and then refed for 18 h with a HCHO diet (see “Experimental Procedures”). Total RNA was extracted and analyzed for the expression of GK, L-PK, FAS, ACC, Spot 14, SREBP-1, and L-CPT I with the indicated 32P-labeled cDNA probes. Representative Northern blots are shown. Ethidium bromide stained 18 S RNA were used as loading controls to normalize the signal obtained for a given gene, n = 2–3/group. B, Western blot analysis of SREBP-1 protein in membrane and nuclear extracts from livers of fasted and refed control and hGK-KO mice. Membrane and nuclear extracts were prepared from 24-h fasted and 18-h HCHO refed control and hGK-KO mice. Proteins from membrane (p SREBP-1c, 125 kDa) and nuclear (m SREBP-1c, 68 kDa) forms of SREBP-1 were separated electrophoretically, transferred to nitrocellulose membrane, and reacted with a SREBP-1 antibody. β-Actin (44 kDa) and Lamin A/C (70 kDa) antibody were used as loading controls to normalize the signal obtained respectively for membrane and nuclear SREBP-1 protein. A representative Western blot is shown, n = 2–3/group.
controls in the liver of fasted hGK-KO mice, despite the lack of GK and for similar levels of SREBP-1c overexpression (Fig. 2). No expression of either GK or FAS was observed after the injection of 10⁹ pfu of either null adenovirus (Ad-null) or SREBP-1c adenovirus (Ad-SREBP-1c). Mice were studied 18 h later. Total RNA was extracted and prepared as described under “Experimental Procedures” for Northern blot analysis. A representative Northern blot (A) hybridized with GK, FAS, and SREBP-1c is shown. The blots are representative of three independent experiments, with 4–6 mice per group. Hepatic G6P (B) and glycogen (C) concentrations were measured in the liver of control and hGK-KO mice, fasted 24 h, non-injected or injected with 10⁹ pfu of Ad-SREBP-1c. Measurements were performed 18 h later, and compared with values obtained for force-fed control and hGK-KO mice. The results are the mean ± S.E., n = 5/group. There was no statistically significant difference in hepatic G6P or glycogen concentrations between control and hGK-KO mice fasted 42 h or overexpressing SREBP-1c.

These results contrast to what was observed in Fig. 1A but can be explained by the fact that when SREBP-1c is overexpressed to higher than physiological levels, a direct induction of FAS gene expression occurs in both control and hGK-KO mice, independently of a GK-dependent increase in hepatic glucose metabolism.

Decreased ChREBP Gene Expression after a High Carbohydrate Refeeding in Livers of hGK-KO Mice—ChREBP was identified in hepatocytes as a major mediator of glucose action on gene expression (20, 33). To determine whether the lack of induction of glycolytic and lipogenic gene expression in livers of hGK-KO mice after HCHO refeeding could be due to an alteration in ChREBP gene expression, we measured by RT-PCR the relative mRNA levels of ChREBP in livers of fasted and refeed control and hGK-KO mice (Fig. 3). In liver of control mice, ChREBP mRNA concentrations were low after a 24-h fast but were increased by about 6-fold upon HCHO refeeding. Similarly, mRNA levels of ChREBP were low in liver of 24-h fasted hGK-KO mice (Fig. 3). However, in contrast to control mice, HCHO refeeding only induced ChREBP gene expression by 2-fold in livers of hGK-KO mice. In addition, we measured the mRNA levels of ChREBP in livers of control and hGK-KO mice previously fasted for 24 h and then injected 18 h with the Ad-SREBP-1c (Fig. 3). In this situation in which glucose metabolism is low (Fig. 2, B and C), there is no difference in ChREBP gene expression between control and hGK-KO mice (Fig. 3), although GK is induced by SREBP-1c overexpression in control mice (Fig. 2A). These results suggest that GK-mediated glucose phosphorylation is necessary for ChREBP gene expression in liver, and that SREBP-1c does not control ChREBP gene expression.

Altered Glucose Metabolism in hGK-KO Hepatocytes Overexpressing SREBP-1c—To determine whether glucose metabolism exerts a potentiating effect on SREBP-1c induction of glycolytic and lipogenic gene expression, we next performed a series of experiments using primary cultures of control and hGK-KO hepatocytes. Cells were infected with 1 pfu/cell of Ad-SREBP-1c and incubated for 24 h at low (5 mM) or high (25 mM) glucose concentrations. The forced expression of SREBP-1c in cultured hepatocytes markedly stimulated the
expression of GK in control hepatocytes at 5 mM, whereas, as expected, no expression of GK was detectable in hepatocytes from hGK-KO mice (Fig. 4A). Similar results were obtained at 25 mM glucose (data not shown).

HKII-III also catalyzes the phosphorylation of glucose to G6P, but markedly differ from GK in terms of glucose affinity, kinetic, and subcellular localization (34). Thus, we also sought to determine whether the expression of any of these hexokinases was increased by SREBP-1c overexpression. Surprisingly, real-time quantitative PCR (RT-PCR) analysis revealed that HKII gene expression was increased by about 80-fold after 24 h of SREBP-1c overexpression (Fig. 4B). This marked induction is independent of the glucose concentration since it was comparable in hepatocytes incubated in either 5 or 25 mM glucose (Fig. 4B). SREBP-1c overexpression had no effect on HKI gene expression (Fig. 4B).

The consequences of SREBP-1c overexpression were also apparent when enzyme activities were measured (Fig. 5A). In control hepatocytes cultured at 5 mM glucose, GK activity increased in parallel with GK gene expression throughout the time course of SREBP-1c overexpression. As expected, no GK activity was measured in hGK-KO–cultured hepatocytes. However, SREBP-1c overexpression at 24 h increased total HK activity to higher levels in hGK-KO than in control hepatocytes (a 10-fold increase compared with a 4-fold increase in controls), probably due to the existence of potential compensatory mechanisms caused by the lack of GK in these hepatocytes (Fig. 5A). Similar results were obtained at 25 mM glucose (data not shown). The higher increase in total HK activity in hGK-KO hepatocytes was not due to higher HKII protein content, which was found to be identical in both control and hGK-KO hepatocytes, 24 h after the adenoviral infection (data not shown). These results suggest that HKII could be preferentially bound to the mitochondria in hGK-KO hepatocytes. This interaction is thought to facilitate glycolysis, allowing HK to use ATP directly generated by the mitochondria, thus ensuring an overall rate of glucose metabolism (34).

To determine the functional consequences of SREBP-1c overexpression on glucose metabolism, G6P, and glycogen content was measured in cells infected with Ad-SREBP-1c (Fig. 5, B and C). Adenovirus-mediated overexpression of SREBP-1c in control hepatocytes cultured in 5 mM glucose induced a time-dependent increase in G6P concentrations. This increase was potentiated at 25 mM glucose since G6P concentrations were about 30% higher than when control hepatocytes were cultured at 5 mM glucose (Fig. 5B). This increase in G6P concentrations in control hepatocytes was concomitant with the increase in GK gene expression and GK activity (Figs. 4A and 5A). In contrast, the absence of GK in hGK-KO–infected hepatocytes had a dramatic effect on their ability to synthesize normal G6P content at both glucose concentrations tested and for comparable levels of SREBP-1c overexpression (Fig. 5B). Indeed, only a small increase in G6P concentration was measured in hGK-KO hepatocytes throughout the time course of the adenoviral infection at either 5 or 25 mM glucose and no potentiating effect of glucose was observed (Fig. 5B). Although HK activity was markedly increased in hGK-KO–infected hepatocytes, G6P levels measured never reached the levels measured in control hepatocytes, suggesting that phosphorylating activity from low $K_m$ HKs is not able to compensate for the lack of GK in hGK-KO hepatocytes.

Glycogen synthesis was low in both control and hGK-KO hepatocytes incubated at 5 mM glucose (Fig. 5C). Glycogen accumulation greatly increased in a time-dependent manner in control hepatocytes incubated at 25 mM glucose (Fig. 5C). In contrast, accumulation of glycogen was markedly diminished in hGK-KO hepatocytes compared with controls cultured at 25 mM glucose, during the time course of the adenoviral infection (Fig. 5C). Although SREBP-1c was overexpressed to similar levels, glycogen deposition in response to increasing doses of glucose was decreased by about 60% in hGK-KO hepatocytes at all times examined (Fig. 5C). These results confirm the pivotal role of hepatic GK in controlling glucose metabolism, and particularly glycogen synthesis in liver.

**Regulation of Glycolytic and Lipogenic Gene Expression in Controlled Culture and hGK-KO Hepatocytes Overexpressing SREBP-1c**—To address the importance of the synergistic action of SREBP-1c and GK-mediated glucose phosphorylation on glycolytic and lipogenic gene induction, we next measured the expression of glucose-regulated genes in SREBP-1c–infected hepatocytes. In control hepatocytes incubated at 5 mM glucose, SREBP-1c overexpression induced the expression of $l$-PK, FAS, ACC, and Spot 14 (Fig. 6). Overexpression of SREBP-1c to similar levels in hGK-KO hepatocytes, incubated in glucose 5 mM, induced to similar levels of the controls FAS, ACC, and Spot 14 gene expression (Fig. 6). In contrast to FAS, ACC, and Spot 14, only a small induction of $l$-PK gene expression by SREBP-1c overexpression was observed in hGK-KO hepatocytes cultured in 5 mM glucose. The level of $l$-PK mRNA never reached the level measured in control hepatocytes at 5 mM glucose (Fig. 6). The induction of $l$-PK (+66%), FAS (+71%), ACC (+118%), and Spot 14 (+94%) measured at 24 h is greatly potentiated in the presence of 25 mM glucose in control-infected hepatocytes (Fig. 6), thereby indicating that both glucose and SREBP-1c, acting in combination, have a strong synergetic effect on the expression of these genes. In contrast, in infected hGK-KO hepatocytes, there is no potentiating effect of glucose on $l$-PK, FAS, ACC, and Spot 14 gene expression at high glucose concentration, demonstrating that the signaling of glucose is lost in absence of GK in these hepatocytes.
ChREBP Gene Expression and X5P Concentrations Are Decreased in Hepatocytes from hGK-KO Mice—To determine whether the loss of the glucose effect observed on the expression of L-PK, FAS, ACC, and Spot 14 in SREBP-1c-infected hGK-KO hepatocytes was due to an alteration in ChREBP expression we measured by RT-PCR the relative mRNA levels of ChREBP in control and hGK-KO hepatocytes cultured in either 5 or 25 mM glucose (Fig. 7A). Expression of ChREBP was decreased by 50% in hGK-KO hepatocytes compared with controls (Fig. 7A). Incubation with a high glucose concentration stimulated ChREBP gene expression by about 1.5-fold in control hepatocytes (Fig. 7A) when GK activity is induced by SREBP-1c overexpression (Fig. 5A). In contrast, in the absence of GK activity, no stimulatory effect of glucose was observed in hGK-KO hepatocytes incubated in the presence of 25 mM glucose and ChREBP mRNA remained lower than what was measured in control hepatocytes incubated at both 5 and 25 mM glucose (Fig. 7A). These results confirm that glucose metabolism via GK is important for the short and long term regulation of ChREBP gene expression in hepatocytes (Fig. 7A).

Because it has been recently suggested that X5P is the glucose signaling compound necessary for ChREBP activation and for the induction of glycolytic and lipogenic gene expression in liver (35), X5P content was measured in 24 h Ad-SREBP-1c-infected control and hGK-KO hepatocytes cultured at 25 mM (Fig. 7B). SREBP-1c overexpression induced a similar increase in X5P concentrations in control hepatocytes cultured at both 5 and 25 mM glucose (Fig. 7B). In contrast, only a moderate increase in X5P concentrations was measured in hGK-KO hepatocytes (Fig. 7B). Based on previously published observations (35), our results suggest that the possible lack of activation of ChREBP by X5P in hGK-KO hepatocytes may explain the loss of the glucose effect observed at high glucose concentrations.

Inhibition of ChREBP Gene Expression by siRNA Prevents Glycolytic and Lipogenic Gene Induction by Glucose in Control Hepatocytes—To determine the direct implication of ChREBP in glucose action on endogenous glycolytic and lipogenic gene expression, we next performed a series of experiments using siRNA to deplete ChREBP gene expression in primary cultures of control hepatocytes. Control hepatocytes were transfected with 200 pmol of either ChREBP or scrambled siRNA and incubated for 24 h at low (5 mM) glucose concentration plus insulin (100 nM). Hepatocytes were incubated in the presence of...
insulin without SREBP-1c overexpression in order to mimic a more physiological context. After ChREBP gene silencing, hepatocytes were incubated for 24 h in the presence of 5 mM glucose. Cells were then incubated with either 5 or 25 mM glucose with 1 pfu/cell of Ad-SREBP-1c. After 6, 10, and 24 h, GK and HK activities at 5 mM glucose (A), G6P (B), and glycogen (C) concentrations were determined. Results are the mean ± S.E. from values obtained from three independent cultures. A, * indicates that total HK activity was greater, at 5 mM glucose, in hGK-KO hepatocytes than in control hepatocytes 10 and 24 h after the adenoviral infection (p < 0.05). B and C, * indicates that the accumulation of G6P and glycogen in control hepatocytes was greater at 25 mM glucose than in 5 mM glucose (p < 0.001). B and C, # indicates that G6P and glycogen concentrations were significantly greater in control hepatocytes than in hGK-KO hepatocytes cultured in 25 mM glucose (p < 0.005). C, § indicates that glycogen concentration at 25 mM glucose is significantly greater in hGK-KO compared with control hepatocytes cultured in 5 mM glucose (p < 0.005).

In non-transfected hepatocytes, incubation with high glucose concentration (25 mM) and insulin stimulated ChREBP gene expression by 3.5-fold (Fig. 8A). Interestingly, glucose alone or low glucose plus insulin had no stimulatory effect on ChREBP gene expression (Fig. 8A). These results confirm, as previously observed (Fig. 7A), that glucose metabolism is necessary for the induction of ChREBP gene expression in hepatocytes. In contrast, ChREBP gene induction by high glucose and insulin concentration was reduced by 60% when control hepatocytes were transfected with ChREBP siRNA (Fig. 8A). In fact, under those conditions, the stimulatory effect of glucose was no longer observed, because ChREBP mRNA levels were similar in ChREBP siRNA-transfected hepatocytes cultured at both 5 or 25 mM glucose with insulin (Fig. 8A). ChREBP gene expression was appropriately induced by high glucose and insulin concentration in hepatocytes transfected with scrambled siRNA (Fig. 8A).

We next measured the expression of glucose-regulated genes by RT-PCR. In non-transfected or scrambled siRNA transfected hepatocytes, the induction of L-PK (+166%) (Fig. 8B), FAS (+188%) (Fig. 8C) and ACC (+200%) (Fig. 8D) was greatly potentiated in hepatocytes cultured with 25 mM glucose and insulin compared with those incubated in the presence of 5 mM glucose plus insulin. In contrast, in ChREBP siRNA-transfected hepatocytes cultured at 25 mM glucose plus insulin, only a marginal potentiating effect of glucose on L-PK (+11%) (Fig. 8B), FAS (+5%) (Fig. 8C), and ACC (+15%) (Fig. 8D) gene expression was observed. Indeed, relative mRNA levels of these genes were equivalent to those measured in control hepatocytes incubated at 5 mM glucose with or without insulin. The effect of ChREBP siRNA was specific to L-PK, FAS, and ACC because insulin-dependent genes, SREBP-1 (Fig. 8E) and GK (Fig. 8F) were normally induced by insulin in ChREBP siRNA-transfected hepatocytes, regardless of the glucose concentration. In addition, both precursor and mature forms of SREBP-1c protein were appropriately expressed in ChREBP siRNA-transfected hepatocytes compared with both control and scrambled transfected hepatocytes (data not shown), demonstrating that the loss of glucose effect observed is exclusively due to ChREBP gene silencing.

Finally, we investigated whether the inhibition of ChREBP gene expression by siRNA had functional effects in terms of lipid synthesis. Culture for 24 h in the presence of 5 mM glucose plus insulin did not lead to lipid accumulation in scrambled siRNA-transfected hepatocytes (Fig. 9). Similar results were obtained with ChREBP siRNA (data not shown). In contrast, in
the presence of 25 mM glucose plus insulin, red-colored lipid droplets were clearly visible in scrambled siRNA-transfected hepatocytes. In contrast, lipid accumulation was largely prevented in ChREBP siRNA-transfected hepatocytes cultured at 25 mM glucose plus insulin (Fig. 9). These findings demonstrate that the decreased in ChREBP gene expression in control hepatocytes partially preclude lipid synthesis even in the presence of high glucose concentration. All together our results demonstrate the direct implication of ChREBP in glucose action on endogenous glycolytic (L-PK) and lipogenic (FAS, ACC) gene expression in hepatocytes.

**DISCUSSION**

Although SREBP-1c has emerged in the past years as a major mediator of insulin action on glycolytic and lipogenic gene expression (7), the extent to which glucose metabolism contributes to its transcriptional effect has not been directly demonstrated. By making use of hepatic GK-KO mice (6), we have shown that glucose metabolism via GK is of primary importance for the maximal induction of glycolytic and lipogenic gene expression in liver. Indeed, in the absence of GK, glycolytic and lipogenic genes are not induced after HCHO refeeding in livers of hGK-KO mice, even in the presence of normal levels of nuclear SREBP-1c protein.

**Effect of Adenovirus-mediated Overexpression of Mature SREBP-1c on GK and HKII Gene Expression**—By overexpressing a dominant positive form of SREBP-1c in control and hGK-KO hepatocytes cultured at either 5 or 25 mM glucose, we were able to examine the relative contribution of glucose metabolism to SREBP-1c action. We found, as previously reported in rat studies (11, 32), that forced expression of a mature form of SREBP-1c induces GK gene expression and activity both in vivo and in cultured hepatocytes from control mice. Surprisingly, we also showed that overexpression of SREBP-1c stimulates the expression of HKII in cultured hepatocytes from control and hGK-KO mice, but not the one of HKI. Recent studies also reported the stimulation of HKII gene expression by SREBP-1c in rat contractile myotubes (36) and human muscle cells (37), but to our knowledge, it is the first report of an insulin-like effect on hepatic HKII gene expression. The fact that putative SRE elements have been identified on the promoter sequence of the rat HKII gene (38) is in agreement with our data, but contrasts with the study of Sebastian et al. (39) in which only transgenic mice overexpressing SREBP-1a, but not SREBP-1c, showed markedly elevated levels of HKII mRNA in liver. This could be caused by the fact that SREBP-1a is a more potent transcriptional activator than SREBP-1c (40), and when
SREBP-1a is overexpressed to higher than physiological levels, it may interact with genes that are not usually its targets. Our study together with the one of Sebastian et al. (39) indicates that the isoforms of SREBP-1 may have an important role in regulating expression of HKII in liver. 

Altered Glucose Metabolism in hGK-KO Hepatocytes Overexpressing SREBP-1c—We have shown that GK is critical for the increased synthesis of G6P and glycogen in response to glucose and SREBP-1c. When SREBP-1c is overexpressed, hGK-KO hepatocytes cultured in the presence of 25 mM glucose, show a marked impairment in their ability to synthesize glycogen even when total HK activity is increased to higher levels than in control hepatocytes. Previous studies have demonstrated that adenovirus-mediated overexpression of GK, but not of HKI, has a potent effect on glycogen synthesis in primary cultured hepatocytes (5, 41), and that only G6P produced by overexpressed GK is glycogenic because it effectively promotes the activation of glycogen synthase. It is clear from our studies that the low $K_m$ HKs did not fully compensate the absence of GK for the normal synthesis of G6P and glycogen in response to high glucose concentration. Thus, the ability of the hepatocytes to efficiently synthesize G6P and glycogen is directly dependent on GK activity and not on HK activity. These data are consistent with studies in which hepatic GK was overexpressed in transgenic mice (2–4) and further support a key role for hepatic GK in maintaining glucose homeostasis. In addition, the recent finding that allosteric activators of hepatic GK improve glucose tolerance and increase hepatic glucose uptake (42) confirms that GK has a high control strength on hepatic glucose metabolism (43).

Synergistic Action of Glucose Metabolism via GK and SREBP-1c on Gene Expression—SREBP-1c has been previously identified as a major mediator of insulin action on lipogenic gene expression as revealed by knockout and transgenic mouse studies (7). Indeed, SREBP-1c knockout mice have an impaired ability to induce lipogenic gene expression after a HCHO refeeding (44) and in transgenic mice that specifically overexpress SREBP-1c in liver, the rate of lipogenesis increases as well as the level of lipogenic gene expression (40, 45). The fact that SREBP-1c, when overexpressed, is able to induce ACC, FAS, or Spot 14 to comparable levels in control and hGK-KO hepatocytes cultured in 5 mM glucose or FAS in the liver of fasted control and hGK-KO mice, suggests a direct stimulatory action of SREBP-1c. In a physiological situation (i.e. refeeding on an HCHO diet) the direct action of SREBP-1c may still occur but, in the absence of glucose metabolism, it is not sufficient by itself to induce glycolytic and lipogenic gene expression. Indeed, even under overexpression conditions, the effect of SREBP-1c on lipogenesis may still occur but, in the absence of glucose metabolism, it is not sufficient by itself to induce glycolytic and lipogenic gene expression.
this activation. The first possibility is the recruitment of a potential glucose-responsive transcription factor that would mediate the glucose signal and act in synergy with SREBP-1c. The recent identification and purification of ChREBP, based on its capacity to bind to the ChoRE of the L-PK promoter (20) has shed light on the possible mechanism whereby glucose affects gene transcription. We have therefore addressed whether the induction of glycolytic and lipogenic gene expression in our experiments could be accounted for by the dual effect of SREBP-1c overexpression and of endogenous ChREBP expression. We found that glucose stimulates the expression of ChREBP in control hepatocytes (both in vivo and in vitro), as also recently demonstrated in INS-1 cells (52) and in 3T3-L1 adipocytes (53). Our results provide the first direct evidence that GK, and consequently glucose metabolism, are necessary for the appropriate expression of ChREBP in hepatocytes. More importantly, decreased ChREBP gene expression in control hepatocytes using small interfering RNA results in a loss of glucose effect on endogenous glycolytic (L-PK) and lipogenic (FAS, ACC) gene expression, demonstrating the direct implication of ChREBP in glucose-induced gene induction by glucose. These results are in agreement with other studies (33, 35, 52) in which ChREBP was described as essential for L-PK gene transcription. However, our data demonstrate for the first time the direct implication of ChREBP in mediating glucose effect on endogenous lipogenic genes such as FAS and ACC in hepatocytes and in the process of lipid synthesis. Our data reinforce the idea that ChREBP plays a crucial role in regulating short term carbohydrate metabolism and fat synthesis.

The discovery of ChREBP and its potential role in glucose action raises the question of the intracellular signaling pathway that is responsible for its activation by glucose. It has been recently proposed that the activity of ChREBP requires a mechanism of phosphorylation/dephosphorylation, which is determined by the relative activity of protein phosphatase 2A (PP2A), regulated by X5P concentrations (35). We have shown that increased glucose metabolism via GK is necessary for the generation of this signal. Indeed, the decrease of glucose metabolism and X5P concentrations in hGK-KO hepatocytes cultured at 25 mM glucose could explain the loss of glucose effect...
In conclusion, we have clearly demonstrated that glucose metabolism via GK is necessary for the transcriptional effect of SREBP-1c on 1-PK, FAS, ACC, and Spot 14 gene expression, further underlying the key role of hepatic GK in this nutritional induction pathway. Moreover, we have demonstrated that ChREBP plays an essential role in the regulation of 1-PK but also of genes involved in lipid metabolism such as FAS and ACC. Finally, our data support the hypothesis that glycolytic and lipogenic gene expression is synergistically regulated by SREBP-1c and glucose acting via ChREBP.

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REFERENCES


FIG. 9. Effects of selective gene silencing of ChREBP on lipid accumulation in cultured mouse hepatocytes. After plating, control hepatocytes were cultured for 24 h in the presence of 5 mM glucose. Hepatocytes were then transfected with 200 pmol of ChREBP or scrambled siRNA and incubated for 24 h with 100 nM insulin and 100 mM dexamethasone, in the presence of 5 mM glucose. After ChREBP gene silencing, hepatocytes were incubated in the presence of 5 or 25 mM glucose with 100 nM insulin and 100 mM dexamethasone. After 24 h, hepatocytes were washed extensively, fixed, counterstained with 4',6-diamidino-2-phenylindole (blue, for nuclei) and stained for the presence of lipid droplets. Microscopic and fluorescent microscopic views of hepatocytes at a magnification of ×200 are shown.
Hepatic Glucokinase Is Required for the Synergistic Action of ChREBP and SREBP-1c on Glycolytic and Lipogenic Gene Expression
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