c-Myc is Required for the Glucose-mediated Induction of Metabolic Enzyme Genes

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Running Title: c-Myc and Glucose-regulated Gene Expression

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Abstract

Glucose exerts powerful effects on hepatocyte gene transcription by mechanisms that are incompletely understood. c-Myc regulates hepatic glucose metabolism by increasing glycolytic enzyme gene transcription while concomitantly decreasing gluconeogenic and ketogenic enzyme gene expression. However, the molecular mechanisms by which c-Myc exerts these effects is not known. In this study, the glucose-mediated induction of L-type pyruvate kinase and glucose 6-phosphatase mRNA levels was diminished by maneuvers involving recombinant adenoviral vectors that interfere with (i) c-Myc protein levels by antisense expression or (ii) c-Myc function through a dominant-negative Max protein. These results were obtained using both HL1C rat hepatoma cells and primary rat hepatocytes. Further, a decrease in c-Myc abundance reduced glucose production in HL1C cells, presumably by decreasing glucose 6-phosphatase activity. The repression of hormone-activated phosphoenolpyruvate carboxykinase gene transcription by glucose was not affected by a reduction in c-Myc levels. The basal mRNA levels for L-pyruvate kinase and glucose 6-phosphatase were not altered to any significant degree by adenoviral treatment. Furthermore, adenoviral overexpression of the c-Myc protein induced glucose 6-phosphatase mRNA in the absence of glucose stimulation. We conclude that multiple mechanisms exist to communicate the glucose-derived signal, and that c-Myc has a key role in the hepatic glucose signaling pathway.
Introduction

Insulin and glucose act jointly to influence glucose homeostasis by altering hepatic gene expression patterns. Insulin increases glucokinase (GK) gene transcription and protein levels in hepatocytes (1). The phosphorylation of glucose by GK leads to increased glucose flux and metabolism, generating signaling metabolites that modify the gene expression profile of the liver (2). For example, the L-pyruvate kinase (L-PK) and glucose 6-phosphatase (G-6-Pase) genes (encoding key glycolytic and gluconeogenic enzymes, respectively) are stimulated by increases in hepatic glucose metabolism (3-5). Conversely, glucose metabolism can also negatively regulate gene transcription, exemplified by the repression of hormone-activated phosphoenolpyruvate carboxykinase gene promoter activity [PEPCK; refs. (6,7)]. Although glucose metabolism modulates the gene expression patterns of key glycolytic and gluconeogenic enzymes, the signaling mechanisms and coordinating transcription factors involved are not fully characterized.

One transcription factor that is a candidate for establishing hepatic glucose-dependent gene expression patterns is the basic, helix-loop-helix leucine-zipper (bHLH-LZ) transcription factor c-Myc (8,9). The Myc family of proteins (e.g., c-, N-, L-Myc, USF, Max, Mad, etc.) participates in the control of proliferation, growth and differentiation, apoptosis, and metabolism (10,11). c-Myc binds DNA by interacting with the E-box sequence CACGTG (and other related non-canonical sites), as a heterodimer with Max, another bHLH-LZ protein. The Myc/Max heterodimer activates or represses transcription depending on the target gene (11,12). Sequences very similar to the E-box are present in
the carbohydrate response elements of the L-pyruvate kinase and S14 gene promoters (3,13,14).

A three-fold overexpression of c-Myc in the liver of transgenic mice, driven by the PEPCK promoter, increases hepatic glucose metabolism and storage, presumably due to the substantial rise in GK activity (no increase in HK I activity was detected) ref. (9). The expression of glucose transporter 2 (GLUT2), L-PK, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) all increase, as well as liver glycogen content, indicating that hepatic glucose metabolism is enhanced. Furthermore, in c-Myc overexpressing animals treated with streptozotocin, and therefore lacking insulin, glucose and ketone production decrease (8). These changes are accompanied by a decrease in mRNA levels for PEPCK and carnitine palmitoyl transferase (CPT) I and II. Importantly, this hepatic elevation in c-Myc protein levels in vivo is sufficient to nearly normalize fasting serum glucose levels and thus prevent streptozotocin-induced diabetes (8). Taken together, these results are consistent with c-Myc playing a major role in the control of hepatic glucose metabolism in vivo. However, the molecular mechanisms mediating these changes have not been elucidated.

In the present study, we decreased the abundance of the c-Myc protein using a recombinant adenovirus that expresses antisense c-myc RNA (AdCMV-ASmyc) to test whether this transcription factor is required for glucose-regulated gene expression in hepatocytes. Cells treated with AdCMV-ASmyc had 50% less c-Myc protein than cells treated with an equal amount of a control adenovirus. This reduction in c-Myc was
sufficient to block the glucose-stimulated expression of glucose-responsive genes in both HL1C rat hepatoma cells and rat primary hepatocytes. Further, a recombinant adenovirus expressing a dominant-negative Max protein mimicked the effect of AdCMV-AS\textit{myc}.

Reducing c-Myc levels decreased glucose production in HL1C hepatoma cells, presumably by decreasing G-6-Pase activity. In addition, treatment with a recombinant adenovirus that expresses c-Myc increased G-6-Pase gene expression when cells were incubated in 2 mM glucose, partially mimicking the effect of 20 mM glucose. Finally, repression of hormone-activated PEPCK gene promoter activity by glucose was not alleviated by a reduction in c-Myc protein, suggesting multiple pathways for glucose signaling.
Experimental Procedures

Cell Culture—The HL1C rat hepatoma cell line contains the PEPCK promoter sequence (from -2100 to +69, relative to the transcription start site) ligated to the chloramphenicol acetyltransferase (CAT) reporter gene (15). The culture of HL1C cells, and the assay for measurement of CAT activity have been described (16,17).

Primary Hepatocytes—Hepatocytes from overnight-fasted or ad libitum fed male Wistar rats (250-350g) were isolated using the collagenase perfusion method as described previously (18). Cells were allowed to attach for 3hr in hepatocyte medium (glucose-free DMEM [Gibco] supplemented with 15 mM HEPES, 33 μM biotin, 40 μM phenol red, 1 mM sodium pyruvate, 17 μM pantothenate, 14 mM sodium bicarbonate, 5 x 10^4 U/L penicillin, 5 x 10^4 μg/L streptomycin, 4 mM L-glutamine, 50 mg/L gentamicin, 2.5 mg/L fungizone) plus 5.5 mM glucose, 10% FBS, 0.5 μM dexamethasone and 10 nM insulin. Recombinant adenovirus was added and allowed to attach to the cells for one hour. The medium containing adenovirus was aspirated and cells were then incubated for 24 hr in fresh hepatocyte medium supplemented with 0.2% BSA and 2 mM glucose. Thus, insulin and FBS were removed from the media for 24 hr, and cells were kept in 2 mM glucose for 24 hr prior to overnight treatment with 20 mM glucose. Preparation of hepatocytes from ad libitum fed rats was as described above for fasted rats, with the exception that the cells were exposed to 10 nM insulin and 5% FBS along with 2 mM glucose for 24 hr after the 3 hr attachment period. The cells were then washed three
times with Dulbecco’s PBS, and re-incubated in hepatocyte medium (without insulin or FBS) for treatment with 2 or 20 mM glucose overnight.

**Construction, Preparation and Use of Recombinant Adenoviruses**—A 500 bp cDNA generated by RT-PCR, and corresponding to nucleotides 2169-2569 (the entire exon 1) and 2570-2670 (100 bp of exon 2) of the rat c-myc gene (GenBank Accession: Y00396) was subcloned in a 3’-5’ orientation into the adenovirus vector pACCMV.pLpA. The insert was sequenced using the dideoxy nucleotide method for verification of correct orientation and sequence and subsequently was used to generate a recombinant adenovirus (AdCMV-ASmyc) as previously described (19). The resulting virus was plaque-purified and used to treat 50% confluent HL1C cells in 10 cm dishes at varying titers. Primary hepatocytes at 80% confluence were treated in 60 mm collagen coated dishes as described in the figure legends. A virus containing the bacterial β-galactosidase gene (AdCMV-βGal) was used as a control (20). After a 60-90 min incubation with adenovirus, the cells were placed in fresh medium, and harvested 40 hr later.

**RNA Isolation**—Total RNA was isolated from HL1C cells and primary hepatocytes using TRI-reagent (Molecular Research Center) according to the manufacturer’s protocol.

**Semi-quantitative RT-PCR Assay**—The reverse transcription (RT) reaction contained 1 µg total RNA, 1 µl random primer (50 µM, Applied Biosystems), 1X reverse transcriptase buffer, 10 U MMLV reverse transcriptase (Promega) in a total volume of 20 µl. The RNA and primer were annealed by heating to 72°C and slowly cooled to room
temperature before the reverse transcriptase was added and the reaction was allowed to proceed at 42°C for 1 h. The RT reaction was then diluted to 100 µl with RNase-free water. The polymerase chain reaction contained 1X Taq polymerase buffer (Promega), 0.5 µl of 20 pmol/µl of each of the primer pair, 200 µM dNTPs, 0.25 µl HotStart-Taq polymerase and 2.5 µl of the RT reaction. The primers for each gene were selected using the PRIMER3 program (MIT) so that each PCR product was approximately 300 bp and each primer had an annealing temperature of 60°C. The primers (upstream and downstream, respectively) used were: for G-6-Pase, 5′ GTGGGTCC-TGGACACTGACT, 5′ CAATGCCTGACAAGACTCCA; for pyruvate kinase, 5′ AACCTCCCCACTCAGCTACA, 5′ TGCTCCACTTCTGTCACCAG; for β-actin, 5′ AACACCCCGCATGTACGAT, 5′ GAACCGCTATTGCGATAGT; and for cyclophilin, 5′ TGGTGCAAGTCCATCTACG, 5′ AAAATGCCCGCAAGTCAAAG. The number of cycles used in the PCR was determined empirically for each primer pair to be within the linear range of amplification. In addition, the amount of RNA used for the RT reaction (1 µg) was determined to be in the linear response range when comparing RNA input to PCR product abundance (data not shown). Furthermore, the sequence of the PCR products was verified by dideoxy sequencing (Sequenase). The PCR reaction was as follows: 15 min at 95°C, followed by 25 to 30 cycles of 30 sec at 95°C, 30 sec at 62°C, and 30 sec at 72°C, followed by a 5 min extension at 72°C. PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. The abundance of the PCR products was quantitated using a Kodak EDAS 290 gel documentation system and NIH/Scion image software. We have validated this assay by finding that the relative abundance of cyclophilin mRNA was linear from 0.1 to 1.0 µg of input RNA (r=0.995)².
Thus, this semi-quantitative RT-PCR assay has a high degree of precision and results in a linear response with respect to input RNA concentrations over at least one order of magnitude.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared as a modification of the procedure described previously (21). The protein concentration was determined using the BCA assay (Pierce Chemical Company) with bovine serum albumin as the standard.

**Western Blots**—Twenty µg of nuclear extract were mixed in sample buffer containing 2% SDS, 100 mM β-mercaptoethanol, 60 mM Tris-HCl, pH 7.8, and 0.01% bromophenol blue. This mixture was boiled in a water bath for 5 minutes, and loaded onto 8% polyacrylamide gels (iGels, Gradipore). The proteins were electrophoresed for 90 minutes at 40 mA, and transferred to PVDF membrane (Pierce Chemical Company) overnight at 12 V in a transfer buffer containing 10% ethanol, 25 mM Tris base, and 192 mM glycine. After blocking with 5% non-fat dry milk in tris-buffered saline (TBS) solution for one hour, the primary antibody (anti-c-Myc, 1:1000 Research Diagnostics; anti-tubulin, 1:5000, a gift from Dr. Kevin Brown) was incubated at room temperature with the membrane for four hours with continuous shaking. The membranes were then washed three times for five minutes each with TBS. After washing, the secondary antibody (anti-rabbit coupled to horse radish peroxidase, Kirkegaard & Perry Laboratories, Inc.) was applied at 1:20,000 and allowed to bind for two hours at room temperature with continuous shaking. The membranes were again washed three times for five minutes. After the third wash, the membranes were exposed to the SuperSignal
chemiluminescent reagent (Pierce) for one minute. Equal protein loading was confirmed by either ponceau S (Sigma) staining of the PVDF membrane after transfer, or by immunoblotting for the abundance of tubulin. Densitometry was performed using Scion image software (NIH).

**Transient Transfection and Dual Luciferase Assay**—Transfections of HL1C rat hepatoma cells were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were first treated with AdCMV-βGal or AdCMV-ASmyc at 50 pfu/cell. Twenty four hours after viral transduction, the cells were transfected using 5 ug of either the pMyc-TA-Luc or the pTA-Luc vectors (Clontech), and with 1 ug of pRL-TK as a control for transfection efficiency (Promega). These cells were harvested after an additional 24 h incubation and luciferase assays were performed in cell lysates using the Dual-Luciferase Reporter Assay System (Promega) in a TD-20/20 luminometer (Turner Designs).

**Glucose Production Assay**—HL1C cells at 50% confluence were treated with AdCMV-GKL and either AdCMV-βGal or AdCMV-ASmyc. Twenty-four hours after viral transduction, the cells were treated overnight with 2 or 20 mM glucose. The media was then replaced with glucose-free Dulbecco’s modified essential medium and glucose production measured essentially as described using a glucose assay kit (Sigma 510-A) (22,23). The medium used for measurement of glucose production was modified slightly to include 1 mM glycerol, 2 mM pyruvate, and 20 mM lactate. The pH of the medium was 7.4 and did not contain phenol red.
**Statistical Analysis**—All results are expressed as means ± standard errors (SE). Data analysis was performed using the statistics module of Microsoft Excel Version 9.0 (Microsoft Corp.). Statistical significance in the form of a two-tailed t-test was rejected at $p > 0.05$. 
Results

**AdCMV-ASmyc produces an antisense c-myc mRNA** A recombinant adenovirus (AdCMV-ASmyc) was constructed to produce an antisense c-myc mRNA. HL1C rat hepatoma cells were transduced with AdCMV-ASmyc or a control adenovirus, AdCMV-βGal (20), and the effect of increasing the moi from 12.5 to 50 pfu/cell on mRNA abundance was assessed by RT-PCR (Fig. 1). Elevation of the viral titer augmented the steady state mRNA levels of antisense c-myc and β-galactosidase.

**AdCMV-ASmyc decreases c-Myc protein levels and transcriptional activity** HL1C cells were transduced with either AdCMV-βGal or AdCMV-ASmyc at 50 pfu/cell to test whether antisense c-myc mRNA would decrease the abundance of c-Myc protein. At 18 hours post-transduction, nuclear protein extracts were harvested and the relative amount of c-Myc was determined by immunoblotting (Fig. 2A and 2B). Treatment with AdCMV-ASmyc depleted c-Myc protein levels by ~50% (n = 3, p<0.05) relative to cells treated with AdCMV-βGal. This reduction is similar to the adenoviral-mediated antisense reduction of p300 reported by Kolli et al. (24). We have subsequently found that AdCMV-ASmyc decreases c-Myc levels by at least 50% in several different culture systems. As a control, HL1C cells were treated with either AdCMV-βGal or AdCMV-ASmyc for 24 h and were then transiently transfected with a plasmid bearing a luciferase reporter gene driven by a minimal promoter (pTA-Luc, Clontech), or a similar construct with the addition of an E-box upstream of the minimal promoter (pMyc-TA-Luc, Clontech; Fig. 2C). The presence of the E-box element, which binds to c-Myc, gives a measure of c-Myc transcriptional activity. While cells transfected with pTA-Luc had
negligible reporter activity (data not shown), those transfected with pMyc-TA-Luc yielded robust luciferase activity. Furthermore, cells treated with AdCMV-AS\textit{myc} and transfected with pMyc-TA-Luc displayed significantly reduced promoter activity compared to cells treated with AdCMV-\textit{β}Gal and transfected with the same reporter plasmid. Together, these results demonstrate that treatment with AdCMV-AS\textit{myc} leads to decreased c-Myc protein levels and transcriptional activity.

\textit{AdCMV-AS\textit{myc} blunts the glucose-stimulated expression of the L-pyruvate kinase and G-6-Pase genes} Glucose metabolism generates a signal that potently affects hepatic gene transcription, and c-Myc has been implicated in the control of glucose-regulated gene expression (2,9,25). Therefore, HL1C cells were transduced with AdCMV-AS\textit{myc} to determine whether the glucose-mediated induction of the L-PK or G-6-Pase genes requires the full complement of endogenous c-Myc protein expression. HL1C cells do not express GK, a feature shared with many hepatoma cell lines (26). Thus, GK was expressed via a recombinant adenovirus to increase glucose flux and metabolism. HL1C cells expressing GK, and treated with 20 mM glucose, display a 2- and 4-fold increase in the mRNAs for the L-pyruvate kinase and G-6-Pase genes, respectively when compared to control cells (Fig.3). These observations are consistent with previous findings wherein glucose decreased PEPCK gene promoter activity in the same cell system (6). Treatment with AdCMV-AS\textit{myc} blunted the glucose-mediated increase in the mRNA levels of L-PK and G-6-Pase in a concentration dependent manner in HL1C cells, while treatment with AdCMV-\textit{β}Gal over the same concentration range had no significant effect on the expression of these genes (Fig. 3).
After observing these effects in HL1C cells, we carried out similar experiments in primary hepatocytes isolated from overnight-fasted rats. In this system, insulin levels, and thus GK levels, are quite low. To provide an increased glucose flux, GK was expressed by adenoviral transduction, which allows us to effectively separate the glucose-signaling pathway from the insulin-signaling pathway. Subsequent addition of 20 mM glucose to these cells increased G-6-Pase gene expression by 4-fold when compared to incubation in 2 mM glucose. However, treatment with AdCMV-AS\textit{myc} blocked the glucose-mediated induction, while AdCMV-\textit{β}Gal had no significant effect (Fig. 4 A). L-PK gene expression was also repressed in rat primary hepatocytes in the presence of AdCMV-AS\textit{myc}, although not as dramatically as in HL1C hepatoma cells, as it did not reach statistical significance (p=0.07) (Fig 4B). Taken together, these data demonstrate that a 50% reduction in c-Myc protein levels is sufficient to inhibit glucose-regulated gene expression in HL1C rat hepatoma cells and rat primary hepatocytes.

\textit{Expression of a dominant-negative Max protein inhibits the glucose-mediated induction of G-6-Pase} We determined whether disabling the ability of the Myc-Max transcription complex to bind DNA had the same effect as reducing c-Myc protein levels on the glucose-mediated induction of the G-6-Pase gene. In these experiments, primary hepatocytes from fed rats were used to take advantage of high endogenous levels of glucokinase, thereby alleviating the need for adenoviral replacement of the enzyme. This cell culture system displays robust glucose-stimulated gene expression similar to our previous model systems described above and is the predominant system used in the study of glucose-mediated gene expression (3,13). We note that AdCMV-AS\textit{myc} also
decreases the abundance of G-6-Pase mRNA is this system (data not shown). An adenovirus expressing a dominant negative, flag-tagged Max protein (AdCMV-AMax), wherein the basic region of Max is replaced with an acidic domain (27), was used to evaluate the necessity of the heterodimer complex. The AMax protein heterodimerizes with c-Myc generating a protein-protein interaction that is thermodynamically more stable than the association of c-Myc with endogenous Max, and the AMax-Myc complex is unable to bind to (27). Adenoviral-mediated expression of the AMax protein in primary rat hepatocytes inhibited the induction of G-6-Pase gene expression by glucose metabolism (Fig. 5). We conclude that a functional Myc-Max complex is necessary for maximal glucose-stimulated expression of the G-6-Pase gene.

**AdCMV-ASmyc blocks glucose production in HL1C rat hepatoma cells**—Since AdCMV-ASmyc blunts the glucose-mediated expression of pyruvate kinase and G-6-Pase, we determined if treatment with this adenovirus had an affect on cellular metabolism. We saw no effect on lactate production (data not shown), perhaps because the effect of AdCMV-ASmyc on PK mRNA was relatively small compared to that of G-6-Pase (Figs. 3 and 4). G-6-Pase catalyzes the removal of the phosphate group from glucose 6-phosphate as the terminal step in gluconeogenesis. We sought to determine whether reducing c-Myc levels would interfere with the ability of HL1C rat hepatoma cells to produce glucose. HL1C cells transduced with AdCMV-GKL and either AdCMV-βGal or AdCMV-ASmyc were used for this purpose. Glycerol was added to the medium as a gluconeogenic substrate to bypass the PEPCK reaction (conversion of oxaloacetate to phosphoenolpyruvate) since the gene encoding this enzyme is known to
be repressed by glucose metabolism (6,7). Under these conditions, glycerol enters the
gluconeogenic pathway at the triose level, and glucose production should increase with
increasing G-6-Pase activity despite a reduction in PEPCK activity. The cells expressing
AdCMV-ASmyc and treated with 20mM glucose (to stimulate G-6-Pase gene expression)
produced approximately 50% less glucose than did cells expressing AdCMV-βGal and
treated with same concentration of glucose (Figure 6). Therefore, reducing c-Myc
abundance reduces the glucose production capacity of HL1C cells.

Reducing c-Myc protein levels in HL1C rat hepatoma cells does not affect
unstimulated mRNA levels of either the L-PK or G-6-Pase genes One possible
mechanism by which AdCMV-ASmyc may influence gene expression is to alter basal
transcription. Thus, we examined the effect of reducing c-Myc protein levels on L-PK
and G-6-Pase mRNA levels in HL1C cells after an overnight culture in 2mM glucose
(Fig 7). We found that the mRNA abundance of each of these genes was not
significantly different in cells transduced with AdCMV-βGal when compared to cells
transduced with the same moi of AdCMV-ASmyc. These data demonstrate that reducing
c-Myc protein levels does not affect unstimulated mRNA levels of the L-PK and G-6-
Pase genes and suggests that c-Myc participates specifically in the glucose-stimulation of
gene transcription.

Overexpressing c-Myc via a recombinant adenovirus increases the mRNA levels of G-
6-Pase at a non-stimulating glucose concentration Quiescent cells, including
hepatocytes, express very little c-Myc protein. However, c-Myc is clearly involved in the
regulation of glucose metabolism in a variety of cell types (28,29), and in this study, it is evident that endogenous, unstimulated levels of c-Myc are required for glucose-induced expression of the L-PK and G-6-Pase genes. To determine whether overexpression of c-Myc was able to induce the expression of the G-6-Pase gene in the presence of 2mM glucose, the relative levels of G-6-Pase mRNA were assessed after transduction of primary hepatocytes from ad libitum-fed rats with either AdCMV-βGal or AdCMV-c-Myc. Under the conditions used, c-Myc protein is undetectable in extracts from primary hepatocytes treated with AdCMV-βGal, but quite abundant after transduction with AdCMV-c-Myc (Fig.8A). The c-Myc treatment produced a 50% increase in G-6-Pase gene expression from cells incubated in 2 mM glucose (Fig. 8B). However, treatment with AdCMV-βGal at the same moi did not alter expression of the gene. Although the increase in gene expression seen in this experiment is not as dramatic as that induced by 20 mM glucose, it is further evidence that c-Myc regulates the expression of the G-6-Pase gene.

Reducing c-Myc levels does not affect the glucose-mediated repression of the hormone-activated PEPCK promoter. Glucocorticoids and glucagon, via the cyclic AMP signaling pathway, activate the PEPCK gene, leading to hepatic glucose production that maintains plasma euglycemia in the absence of ingested fuel substrates (30). Glucose metabolism provides a signal that leads to transcriptional repression of the PEPCK gene in HL1C cells (6,7). We sought to determine whether diminishing c-Myc protein levels alleviated the glucose-mediated repression of PEPCK promoter activity. HL1C cells contain the PEPCK promoter (-2100 to +69) sequence linked to the chloramphenicol
acetyltransferase (CAT) reporter gene (15). Treating HL1C cells with dexamethasone and 8-(4-chlorophenyl-thio)cAMP produces a 50-fold induction of CAT activity. In the presence of adenovirally-expressed GK and 20mM glucose, this activity is reduced by 80% (6). Depleting c-Myc levels did not reverse the glucose-dependent repression of CAT activity, nor did the control virus lessen the glucose-dependent effects (Fig 9). Surprisingly, the highest moi of AdCMV-ASmyc further repressed promoter activity significantly, while the same moi of AdCMV-βGal did not. These results demonstrate that c-Myc is not necessary for the glucose-mediated repression of the hormone-activated PEPCK promoter, and suggest the existence of multiple glucose signaling pathways.
Discussion

Glucose is a strong regulator of hepatic gene transcription (2,25). Increased glucose metabolism coordinately increases the expression of glycolytic and lipogenic enzyme genes, and decreases the expression of ketogenic and gluconeogenic enzyme genes. c-Myc regulates hepatic glucose metabolism in vivo by increasing the expression and activity of glycolytic enzymes and exerting the reverse effect on those of glucose and ketone production (8,9). In this study, we demonstrate several important observations regarding the role of c-Myc in hepatic glucose metabolism: 1) maneuvers that result in a reduction of c-Myc protein levels were able to blunt the glucose-stimulated induction of G-6-Pase and mRNAs in both HL1C rat hepatoma cells and rat primary hepatocytes; 2) adenoviral expression of a Max dominant-negative protein had the same effect as reducing c-Myc levels; 3) the abundance of c-Myc influences glucose production from glycerol, presumably by altering G-6-Pase activity; 4) the mRNAs for L-PK or G-6-Pase were not affected by treatments resulting in decreased c-Myc protein levels in unstimulated (culture at 2mM glucose) cells; 5) c-Myc overexpression leads to an induction of the G-6-Pase gene in cells cultured with 2 mM glucose; 6) the signal for a glucose-mediated repression of hormone-activated PEPCK is unaffected by diminished c-Myc protein levels.

A three-fold overexpression of c-Myc driven by the PEPCK promoter in the liver of transgenic mice increases GK, PFK2, and L-PK gene expression despite insulin deprivation by streptozotocin treatment (8). These changes in gene expression are sufficient to increase hepatic glucose flux and suppress glucose output from the liver,
thereby protecting the animals from overt diabetes. In the present study, a 50% reduction of c-Myc in rat hepatoma cells and primary hepatocytes blunts the ability of glucose to stimulate expression of the L-PK and glucose 6-phosphastase genes (Figs. 3 & 4). In addition, overexpression of c-Myc partially mimics the glucose effect in primary hepatocytes (Fig. 8). Therefore, our observations are consistent with the transgenic findings. Together, these data indicate that c-Myc is important for controlling hepatic gene expression patterns, and is a key factor in the regulation of gene expression by glucose.

In this study, a dominant-negative Max protein (AdCMV-AMax) was expressed by adenovirus to test whether a Myc-Max heterodimer is required for the glucose-activation of G-6-Pase. Introduction of the AMax protein, which functions as a dominant-negative by allowing heterodimerization but not DNA binding, prevented the glucose-induced rise in G-6-Pase mRNA levels (Fig. 5). This observation suggests that a functional Myc-Max heterodimer is required for the stimulatory glucose effect. How this complex regulates gene transcription upon stimulation with glucose is unknown.

Glucose metabolism increases G-6-Pase mRNA and protein levels (5,31,32). Since reducing c-Myc levels blunted the ability of glucose to stimulate G6Pase mRNA levels (Figs. 3 and 4), we determined whether this affected the ability of the cell to produce and release glucose. We found that decreasing c-Myc levels diminished the glucose production of HL1C cells (Fig. 6). Thus, glucose production can be modified by altering levels of c-Myc.
The physiological significance of glucose exerting a stimulatory effect on G-6-Pase and a repressive effect on PEPCK, both gluconeogenic enzymes, has never been fully understood (6,7). There are several possible explanations for the paradoxical glucose-mediated induction of G-6-Pase. One possibility is that a glucose-mediated elevation in G-6-Pase activity serves as a feedback mechanism to decrease the signal intensity provided by increased glucose flux and metabolism. By this view, the glucose-stimulated increase in G-6-Pase would decrease the abundance of glucose 6-phosphate and other potential signaling metabolites, particularly after the suppressive effect of insulin (33) has diminished towards the end of the fed state. This would limit the extent of glucose signaling and potentiate a transition to the fasted state. One consequence of this arrangement is that an overexpression of G-6-Pase, as seen in Type II diabetes, would lead to a decreased ability of c-Myc and other factors to coordinately regulate hepatic metabolic enzyme genes, and may partially explain the dysregulated hepatic glucose metabolism seen in Type II diabetes (34).

The glucose-mediated induction of the L-PK gene, and presumably other glucose-responsive genes, requires GK expression and glucose metabolism in primary hepatocytes, rat hepatomas and \textit{in vivo}² (31,35). Further, the repression of hormone-activated PEPCK promoter activity is dependent upon the metabolism of glucose, and occurs in the absence of insulin provided GK is expressed (6). Thus, glucose downregulates its own production in the liver via a negative feedback mechanism at the same time that it promotes glucose utilization. However it is not known if the coordinating mechanisms for increasing gene transcription are the same for the repression
of gene transcription. In the present study, we found that reducing c-Myc levels had no
effect on the glucose-repression of the PEPCK promoter (Fig. 9), suggesting that multiple
glucose-signaling pathways exist.

Glucose regulates gene transcription by acting through specific promoter elements,
designated carbohydrate response elements, or ChoREs (3,25). These elements were first
described in the context of the L-PK gene promoter, and require a bHLH-LZ family
member to fully stimulate the gene’s response to carbohydrate (3,36). The L-PK ChoRE
contains two non-canonical E-boxes separated by five base pairs. The E-boxes described
for the glucose-response element of the L-PK gene promoter are different from the c-Myc
family canonical E-box by a single nucleotide (3,13). Also, a conserved carbohydrate
response element consisting of “half E-boxes” (CACG), and documented in the S14 gene
promoter, is necessary and sufficient to regulate transcription in response to glucose (37).
Establishment of the ChoRE lead to the detection, by electrophoretic mobility shift assay,
of a novel ChoRE-binding factor (ChoRF) that still awaits complete characterization
(36,37).

Another candidate for activation of glucose-responsive genes was provided by the
discovery of a novel transcription factor, termed carbohydrate response element-binding
protein (ChREBP), that regulates hepatic L-PK gene expression upon refeeding in rats
(38,39). ChREBP, another bHLH/leucine zipper protein, is regulated by phosphorylation
status, which controls its nuclear localization and DNA binding capabilities (39). In the
presence of glucose, ChREBP is de-phosphorylated, migrates to the nucleus, and
participates in controlling glucose-dependent L-PK gene expression, possibly by binding to the E-box motif present in the L-PK promoter (38,39). Interestingly, the Williams-Beuren syndrome critical region 14 protein (WBSCR14), which is the human homologue of rat ChREBP, heterodimerizes with Mlx, a member of the c-myc family of transcription factors (40). The association, if any, between c-Myc, Max, Mlx and ChREBP/WBSCR14 remains undetermined.

The question that remains unanswered at the present time is whether c-Myc or the Myc-Max heterodimer is a carbohydrate response element binding protein. We found that, unlike pancreatic beta cells (41), glucose does not increase the abundance of c-myc mRNA or protein in hepatocytes3. Assuming that the Myc-Max heterodimer binds directly to ChoREs, there are several explanations regarding the necessity of c-Myc for glucose-stimulated gene expression. These include, but are not limited to, the following. The signal provided by increased glucose metabolism in hepatocytes: 1) promotes a stronger Myc-Max heterodimer interaction; 2) strengthens the ability of the complex to bind to DNA; 3) facilitates contact with co-activators or other constituents of the transcriptional machinery; 4) destabilizes non-Myc-Max heterodimer interactions that would serve to repress the transcriptional activity of glucose-responsive genes. It cannot be ruled out that combinations of these possibilities exist. Another explanation is that Myc-Max acts indirectly, by promoting the expression or activity of other ChoRE-binding factors.
The work of Osthus et al. shows that c-Myc activates glycolytic genes in rat fibroblasts and in vivo in hepatocytes (29). These investigators demonstrated that mRNA levels for the glycolytic enzyme genes, GLUT1, phosphofructokinase-1, glyceraldehyde 3-phosphate dehydrogenase and α–enolase were all upregulated in c-Myc transformed fibroblasts. Further, the same gene expression profile was observed in hepatocytes after mice were infused with an adenovirus expressing c-Myc (29). In addition, they determined which genes are direct c-Myc targets using a chimeric protein that has c-Myc fused to the estrogen receptor ligand-binding domain (MycER). The induction of GLUT1, PFK, and enolase mRNAs in rat fibroblasts increases when the MycER fusion protein is activated by 4-hydroxytamoxifen. This effect is not blocked by the protein synthesis inhibitor cycloheximide, demonstrating that these genes are direct targets of the c-Myc protein (29). c-Myc may then directly regulate the expression of certain glycolytic enzyme genes, and participate, directly or indirectly, in the stimulation by glucose of other metabolic enzyme genes, such as L-PK and G-6-Pase. Indeed, the results of the present study indicate a role for c-Myc in the glucose-mediated stimulation of the L-PK and G-6-Pase genes, but not in the glucose-mediated repression of the hormone-activated PEPCK promoter, suggesting the existence of c-Myc-dependent and independent glucose signaling pathways.

In summary, c-Myc depletion inhibits glucose-dependent gene expression of both the L-PK and G-6-Pase genes, but does not alleviate the glucose repression of hormone-activated PEPCK. We interpret these findings to indicate that multiple signaling pathways exist to communicate the glucose signal to target genes. Also, expressing a
Max dominant negative protein has the same effect as depletion of c-Myc, illustrating the importance of a functional Myc-Max heterodimer for maximal glucose-stimulation of the L-PK and G-6-Pase genes. Furthermore, overexpression of c-Myc mimics the effect of increased glucose metabolism. Taken together, these observations demonstrate an important role for c-Myc in hepatic glucose-mediated gene expression.

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Footnotes

1 The abbreviations used are: GK, glucokinase; L-PK, Liver-type pyruvate kinase; G-6-Pase, G-6-Pase; PEPCK, phosphoenolpyruvate carboxykinase; bHLH-LZ, basic helix-loop-helix leucine-zipper; S14, spot 14; HK I, hexokinase isoform I; GLUT2, glucose transporter isoform 2; PFK2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; CPT, carnitine palmitoyl transferase; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse-transcription polymerase chain reaction; 4-HOTM, 4-hydroxytamoxifen.


3 J.J. Collier and D.K. Scott, unpublished observations.
References

Figure Legends

Fig. 1. **Treatment with AdCMV-ASmyc generates antisense c-myc mRNA.** HL1C cells were treated with either AdCMV-βGal or AdCMV-ASmyc at 12.5, 25, or 50 pfu/cell. The medium was replaced, cells were cultured for an additional 24 hours, and total RNA was collected. The abundance of mRNA expressed by each virus was detected by RT-PCR using the specific primers indicated on the left. Inverse exposures are shown of PCR products separated on agarose gels in the presence of ethidium bromide. Each column represents a series of PCR reactions using the products of a single reverse transcription reaction as the template.

Fig 2. **AdCMV-ASmyc decreases c-Myc protein levels and transcriptional activity in HL1C rat hepatoma cells.** HL1C cells were treated with either AdCMV-βGal or AdCMV-ASmyc at 50 PFU/cell. (A) Nuclear extracts were harvested 18 hours post transduction and the abundance of c-Myc was determined by immunoblotting. (B) A densitometric analysis of the autoradiograms was performed wherein the values were normalized to tubulin. The protein abundance from AdCMV-βGal treated cells was set at 100%. The data are the means ± SE (n = 4, * p < 0.05). (C) HL1C cells were transduced with AdCMV-βGal or AdCMV-ASmyc for 24 h, and then cells were transfected with 5 ug of either pMyc-TA-Luc or the pTA-Luc, and with 1 ug of pRL-TK as a control for transfection efficiency. The plasmid constructs were allowed to express for 24 h before harvesting and measurement of luciferase activities in the cell lysates. RLU, relative light unit, defined as the ratio between Renilla and firefly luciferase activities. Data are
presented as the means (± SE) of three independent experiments, each performed in duplicate (* p<0.05).

Fig. 3. **AdCMV-ASmyc decreases glucose-stimulated gene expression in HL1C cells.** HL1C cells were treated with AdCMV-GKL in the presence of either 2 or 20 mM glucose, or 20 mM glucose with the addition of various amounts of either AdCMV-ASmyc or AdCMV-βGal (12, 25, or 50 PFU/cell). RNA was collected and the relative abundance of pyruvate kinase (A) or G-6-Pase (B) mRNAs determined by RT-PCR. The data are the means (± SE, n=4) of the mRNA abundance normalized to β-actin mRNA levels with the maximal glucose response set at 100% (*, p < 0.05; **, p < 0.01). Representative pictures of ethidium-bromide stained agarose gels with the inverse image are shown (*insets*).

Fig. 4. **AdCMV-ASmyc decreases glucose-stimulated gene expression in primary hepatocytes.** Hepatocytes were isolated from the livers of rats that had been fasted overnight. The cells were treated with AdCMV-GKL in the presence of either 2 or 20 mM glucose. In addition, the indicated groups of cells were treated with various amounts of either AdCMV-ASmyc or AdCMV-βGal (12, 25, or 50 PFU/cell). RNA was collected and the relative abundance of G-6-Pase (A) or pyruvate kinase (B) mRNAs determined using RT-PCR. The data are the means (± SE, n=3) normalized to β-actin with the mRNA abundance of the maximal glucose response set at 100% (*, p < 0.05). Representative inverse images of the PCR products on agarose gels are displayed (*insets*).
Fig. 5. **AdCMV-AMax blunts the glucose stimulation of G-6-Pase mRNA in rat primary hepatocytes.** Primary hepatocytes isolated from *ad libitum* fed rats were treated overnight with various amounts of AdCMV-AMax or AdCMV-βGal (30, 60, or 90 PFU/cell) in the presence of either 2 or 20 mM glucose. (A) A representative immunoblot of the flag-tagged AMax protein (90 pfu/cell) is shown. (B) Shown is a representative photo of PCR products from an inverted image of an ethidium-bromide stained agarose gel (*inset*). The data are means ± SE of four independent experiments with the mRNA abundance normalized to cyclophilin and the maximal glucose response set at 100% (*, p < 0.005).

Fig 6. **AdCMV-ASmyc represses glucose production in glucose-stimulated HL1C cells.** HL1C cells transduced with AdCMV-GKL and either AdCMV-βGal or AdCMV-ASmyc for 24 h were treated with either 2 or 20 mM for an additional 18 h. The cells were washed three times with phosphate-buffered saline, followed by incubation in glucose-free DMEM, supplemented with 1 mM glycerol, 2 mM pyruvate, and 20 mM lactate for 4 h. The medium was removed and glucose was measured as described in the Experimental Procedures Section. Data represent the means (± SE), normalized to total protein, for four independent experiments (* p < 0.01).

Fig. 7. **AdCMV-ASmyc does not affect basal mRNA levels of the L-pyruvate kinase or G-6-Pase genes.** HL1C cells were transduced with various amounts of AdCMV-ASmyc or AdCMV-βgal (12.5, 25, or 50 pfu/cell), and then cultured overnight in 2 mM glucose. Cells were harvested after an additional 24 hrs in fresh medium containing 2mM
glucose. The data are the means of the mRNA abundance of L-PK (A) or G-6-Pase (B) (+ SE) and represent three independent experiments. Representative inverse images of ethidium-bromide stained agarose gels displaying PCR product abundance are shown (insets).

Fig. 8. Overexpression of c-Myc induces the expression of the G-6-Pase gene.
Primary hepatocytes from *ad libitum* fed rats were transduced with AdCMV-c-Myc or AdCMV-βGal (20 or 40 pfu/cell) in the presence of 2 mM glucose. Also, primary hepatocytes from the same preparation were cultured with either 2 mM or 20 mM glucose without adenoviral transduction. (A) A representative immunoblot of c-Myc protein (40 pfu/cell) accumulation is shown. (B) The relative G-6-Pase mRNA abundance was determined by RT-PCR. The means (+ SE) are shown for five independent experiments (*, p < 0.05). An inverse of an agarose gel showing PCR product accumulation is shown (inset).

Fig. 9. AdCMV-ASmyc does not alleviate the glucose-repression of hormone-activated PEPCK promoter activity. HL1C cells were treated with AdCMV-GKL and various amounts of either AdCMV-ASmyc or AdCMV-βGal overnight in serum free medium. The cells were then incubated overnight in control medium or medium containing 0.5 μM dexamethasone (dex) and 100 μM 8CPT-cAMP (cAMP) or dex/cAMP supplemented with either 2 or 20mM glucose. The cells were harvested and chloramphenicol acetyltransferase (CAT) activity in the cell lysates was measured. Shown are the means ± SE of three separate experiments (*, p < 0.05).
Figure 1
Figure 2

A

\[ \text{c-Myc} \quad \beta \text{Gal} \quad \text{AS}_{myc} \]

\[ \text{Tubulin} \quad 55 \text{kD} \quad 68 \text{kD} \]

B

Percent Control (Normalized to Tubulin)

\[ \begin{align*}
\text{AdCMV-} & \beta \text{Gal} \\
\text{AdCMV-AS}_{myc} & \quad \ast
\end{align*} \]

C

E-Box \quad \text{TATA Box} \quad \text{Luciferase}

RLU

\[ \begin{align*}
\text{AdCMV-} & \beta \text{Gal} \\
\text{AdCMV-AS}_{myc} & \quad \ast
\end{align*} \]
Figure 3

A

Percent Maximum Glucose Response

Glucose (mM) 2 20 20 20 20 20 20 20 20

* ASmyc

βGal

B

Percent Maximum Glucose Response

Glucose (mM) 2 20 20 20 20 20 20 20 20

* ASmyc

βGal
Figure 6

The diagram shows the glucose production (mg/dL/μg protein) at different glucose concentrations (2 mM and 20 mM) for two groups: AdCMV-βGal and AdCMV-ASmyc. The error bars indicate the variability in the measurements. The AdCMV-ASmyc group shows a significantly higher glucose production at 20 mM glucose concentration compared to the AdCMV-βGal group.