c-myc Promoter Binding Protein Regulates the Cellular Response to an Altered Glucose Concentration

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ABSTRACT: α-Enolase is a bifunctional gene encoding both a glycolytic enzyme and a DNA binding protein, c-myc binding protein (MBP-1). MBP-1 binds the c-myc promoter and downregulates c-myc transcription. Since these α-enolase gene products have important functions in glucose metabolism and growth regulation, this gene may play a central role in regulating the abnormal proliferative characteristics of transformed cells. To determine the role of α-enolase and MBP-1 in the cellular response to altered exogenous glucose concentration, MCF-7 cells were cultured in low (1 nM), physiological (5 mM), or high (25 mM) levels of glucose. Levels of α-enolase, MBP-1, and c-myc expression were compared to levels of cell proliferation and lactate production. At all glucose concentrations, MCF-7 cells demonstrated an initial increase in MBP-1 expression and a parallel decrease in c-myc transcript levels, which were accompanied by decreased proliferation. Cells grown in low glucose maintained the increased MBP-1 expression through 48 h, resulting in persistently lower rates of proliferation. However, physiologic or high glucose levels resulted in decreased MBP-1 expression, which was associated with increased cellular proliferation and lactate production. In these cells, c-myc mRNA returned to control levels as MBP-1 expression decreased. Cells grown in low glucose demonstrated a dramatic increase in c-myc mRNA at 48 h, which was associated with a loss in c-myc P_2 promoter binding by MBP-1. This suggests that post-translational modifications of MBP-1 likely alter its DNA binding activity. These results demonstrate an important role for MBP-1 in the altered cell proliferation and energy utilization that occur in response to an altered glucose concentration.

Most transformed cells exhibit a dramatic increase in glycolytic metabolism, causing production of lactic acid under aerobic conditions (Warburg effect) (*1, 2*). Changes in the tumor microenvironment may select for cells with a proliferative advantage and resistance to apoptosis and may be responsible for the lack of response of neoplasms to conventional chemo/radiation therapy (*3*). Although the exact mechanism of the Warbug effect is not completely understood, the c-myc oncogene has been implicated due to its common overexpression in transformed cells, its role in stimulating cell proliferation (*4*), and its ability to directly upregulate the transcription of several glycolytic enzymes, including α-enolase (*5*). α-Enolase (48 kDa) catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate in the glycolytic pathway and is directly stimulated by c-myc (*6*). Interestingly, α-enolase mRNA also gives rise to an alternative translation product, c-myc binding protein (MBP-1,[1] 37 kDa), which negatively regulates c-myc transcription by binding to the P_2 promoter (*7*−*9*). This provides an important "feedback control" loop which regulates both c-myc expression and glycolytic activity.

In contrast to α-enolase, MBP-1 is preferentially localized in the cell nucleus. Its potential role as a tumor suppressor is supported by its ability to reduce invasiveness and colony formation of breast cancer cells, suppress tumor formation in nude mice (*10*), and regress lung tumor growth (*11*). The α-enolase gene also maps to a region of chromosome 1, which is often deleted in several human malignancies, including breast carcinoma (*12*). High levels of α-enolase expression in human tumors predict aggressive behavior and poor clinical outcome, suggesting that preferential translation of α-enolase and downregulation of MBP-1 may stimulate tumor growth (*13*).

The relative contribution of endogenous α-enolase and MBP-1 to cell growth and development of the hyperglycolytic phenotype (Warburg effect) is currently unknown. Thus, the study of α-enolase provides a unique opportunity to understand the potential regulatory interactions between cellular metabolism and growth. Since MBP-1 was previously shown to downregulate tumor growth in breast cancer cells, we cultured MCF-7 cells in low (1 nM), physiologic (5 mM), or high (25 mM) concentrations of glucose. Levels of gene expression of α-enolase, MBP-1, and c-myc were compared to cell proliferation and lactate production to determine the role of α-enolase and MBP-1 in the regulation of cell proliferation and development of the hyperglycolytic phenotype. The results of these experiments demonstrate an

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[1] Abbreviations: MBP-1, c-myc binding protein; MCF-7, human breast carcinoma cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT-PCR, real-time polymerase chain reaction; EMSA, electrophoretic mobility shift assay; sem, standard error of the mean.

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important role for MBP-1 in regulating cell proliferation and promoting the hyperglycolytic phenotype in response to altered glucose concentrations.

EXPERIMENTAL PROCEDURES

Cell Culture. MCF-7 cells were purchased from American Type Culture Collection and cultured in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 4 mM glutamine supplemented with 10% charcoal-stripped fetal bovine serum (FBS) and 100 units of penicillin/streptomycin. For experiments, cells were plated and allowed to settle overnight. The next day, cells were washed with glucose-free DMEM and incubated with DMEM containing 1 nM (low), 5 mM (physiologic), or 25 mM (high) glucose supplemented with 10% dialyzed FBS (Invitrogen) and 100 units of penicillin/streptomycin. Cells were harvested at 0 h (before the medium was changed, control) and at various times and used for subsequent experimental analysis.

Cell Proliferation Assay. Cells were plated in a six-well plate at a density of 7.5×10^4 per well at 40% confluence. Cell counts and viability were determined by trypan blue exclusion, counting on a hemocytometer at various time intervals.

Lactate Assay. Cells were plated in a six-well plate at a density of 7.5×10^4 cells per well in 2 mL of medium. The next day, the medium was suctioned off and cells were rinsed with glucose-free, phenol red-free DMEM and incubated in 1 nM, 5 mM, or 25 mM glucose medium (phenol red-free) at 37 °C and 5% CO_2. Medium was collected, and lactate concentrations were determined by a previously described method (*14*). This method monitors the NADH product at 340 nm after the NAD-linked conversion of lactate to pyruvate by lactate dehydrogenase with hydrazine trapping of pyruvate to ensure the reaction goes to completion. One hundred microliters of conditioned medium or various volumes (20−100 μL) of L-lactic acid standard (40 μg/mL) were diluted in a 96-well plate to a final volume of 100 μL. The NAD/glycine buffer solution (pH 9.2, 75 μL) and the lactate dehydrogenase solution (25 μL) were added to each of the standards and sample wells. After incubation for 30 min at 25 °C, absorbance was read at 340 nm and compared to a linear lactate standard curve (2−100 μg/mL). Medium blanks showed negligible absorbance.

Western Blotting for α-Enolase, MBP-1, and c-myc. To prepare protein extracts, cells were plated in a 60 mm culture dish at a density of 5×10^5 per plate and subjected to the experimental protocol as previously mentioned. Cells were collected from each of the three treatment groups and lysed with mammalian extraction reagent (M-PER) (Pierce, Rockford, IL) containing protease inhibitors (Complete Mini, Roche). Thirty micrograms of total cell lysate was separated by SDS−PAGE on a 4 to 15% gradient denaturing gel and electroblotted onto PVDF membranes. The gel transfer efficiency and equal loading of proteins were verified by Ponceau S staining of PVDF membranes. The membranes were blocked for 1 h with 5% nonfat milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) and incubated overnight at 4 °C with an anti-α-enolase (*sc-7455*) or c-myc (*sc-40*) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After being washed with PBS-T, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were visualized using standard chemiluminescence (ECL) methods. Equal loading of proteins was verified by probing the membrane with a mouse monoclonal anti-β-actin primary antibody (Sigma Chemical, St. Louis, MO). The films were scanned with an optical scanner (Epson Expression 1680) and quantified by measuring the density of each band using UNSCAN-IT software (Silk Scientific, Inc., Orem, UT). To correct for possible unequal loading, each band's density was normalized to its β-actin density. To allow for multiple comparisons between gels, each sample was compared to its respective 0 h that was run on the same gel.

Enolase Activity Assay. The enzymatic reaction was performed at 25 °C in a solution containing 100 mM HEPES buffer (pH 7.0), 10 mM $MgSO_4$, 7.7 mM KCl, 10 mM 2-phosphoglyceric acid, and 3 μg of total cell lysate. Conversion of the substrate was assessed in a UV light transparent 96-well plate at 240 nm. A positive control reaction in which pure yeast α-enolase (1 milliunit) was used in place of cell lysate was assessed. Negative control reaction mixtures (blanks) containing no lysate exhibited negligible absorbance. Changes in activity were evaluated by changes in the rate of reaction (V_{max}) and expressed as a percent of the 0 h control.

RT-PCR. Total RNA was extracted from MCF-7 cells using TriZol reagent according to the manufacturer's instructions. RNA purity was determined by the A_{260}/A_{280} ratio and quantified by A_{260}. To prepare cDNA, 2 ng of total RNA was incubated in a reaction mix containing Taqman RT buffer, 5.5 mM $MgCl_2$, a mixture of deoxyNTPs (500 μM per dNTP), 2.5 μM random hexamers, 0.4 unit/μL RNAse inhibitor, and 1.25 units/μL Multiscribe Reverse Transcriptase (Taqman Reverse Transcription Reagents, Applied Biosystems, Foster City, CA). The reaction mixture was first incubated for 10 min at 25 °C to maximize primer−RNA template binding. Reverse transcriptase conversion of RNA into cDNA was performed during a 30 min incubation at 48 °C, immediately followed by a 5 min incubation at 95 °C to stop the reaction and denature the cDNA.

All primers were designed using Primer Express (Applied Biosystems): α-enolase forward primer 5′-ATCGCCAAGGCCGTGAA-3′ and reverse primer 5′-ACGGAGCCAATCTGGTTGAC-3′, c-myc forward primer 5′-CGTCTCCACACATCAGCACAA-3′ and reverse primer 5′-TCTTGGCAGCAGGATAGTCCTT-3′, glut-1 forward primer 5′-GGGTCAGGCTCCATTAGGATT-3′ and reverse primer 5′-CCCAACTGGTCTCAGGTAAAGAA-3′, and β-actin forward primer 5′-TGCCGACAGGATGCAGAAG-3′ and reverse primer 5′-CTCAGGAGGAGCAATGATCTTGA-3′. RT-PCR was performed for a uniform amount of cDNA using the Fast 7500 system (Applied Biosystems). Reaction mixtures were diluted 1:2 with SYBR Green I Master Mix (Applied Biosystems), and amplification by PCR was as follows: one repetition at 50 °C for 2 min, one repetition at 95 °C for 10 min, and 40 repetitions at 95 °C for 15 s and 60 °C for 1 min, representing the melting, primer annealing, and primer extension phases of the reaction, respectively. A no template control reaction was run for each gene to control for DNA contamination of RNA extracts. Following amplification, a dissociation curve was determined to provide evidence for a single reaction product. Message of α-enolase,

c-myc, and glut-1 was compared to the 0 h control to calculate an expression ratio.

Electrophoretic Mobility Shift Assay. To assemble the P_2 promoter of c-myc, a 50 bp oligonucleotide (positions −52 to +2 relative to the P_2 start site on the c-myc promoter) was radiolabeled using [γ-^{32}P]dATP with T_4 polynucleotide kinase. It was annealed to its complement by being heated at 90 °C for 10 min in 20 mM Tris-HCl (pH 7.4) and 10 mM Mg Cl_2 and slowly cooled to room temperature to form a radiolabeled double-stranded probe. Nuclear extracts were prepared as previously described (*15*) from samples taken at 6 or 48 h from 1 nM, 5 mM, or 25 mM glucose. The probe (50 000 cpm) was incubated with 5 μg of nuclear extract in 20 mM Tris-HCl (pH 7.4), 140 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 8% (v/v) glycerol, and 0.2 mM PMSF for 30 min at 37 °C. In some reactions, 150 nM unlabeled competitor MP2 5′-AGGGATCGCGCTGAGTATAAAAGC-CGGTTTTCGGGG-3′, containing the binding site for MBP-1, or nonspecific competitor BEE-1 5′-AGCTGTTCT-GAGTGGGGGAGGGGGCTGCGCCTGC-3′, containing an unrelated consensus sequence, was added before the probe was added to demonstrate specificity. A supershift was performed by crosslinking 5 μg of anti-α-enolase polyclonal antibody to the nuclear extract with a Stratagene UV Stratalinker prior to addition of the labeled probe. The protein−DNA binding reaction mixtures were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel at room temperature in 1× Tris-borate-EDTA. Complexes were visualized by autoradiography.

Data Analysis. All values represent the mean ± the standard error of the mean. Differences between time points and controls were determined by a one-way analysis of variance (ANOVA). When the means of only two experimental groups were compared, a nonpaired *t* test was used. A $p < 0.05$ probability level was used to indicate statistical significance.

RESULTS

Cell Proliferation and Viability. To determine the effect of glucose concentration on cellular proliferation, MCF-7 breast cancer cells were incubated in 1 nM (low), 5 mM (physiological), or 25 mM (high) glucose. Changes in cell proliferation and viability were measured by trypan blue counting of cells. At each glucose concentration, there was a slight increase in cell number after 6 h (Figure 1). Cellular proliferation with 1 nM glucose was significantly slower than proliferation with physiologic or high levels of glucose through the completion of the experiment at 48 h. Trypan blue staining confirmed that the majority [91.8 ± 2.8% (n = 4)] of these cells remained viable at 48 h. On the other hand, with a physiologic (5 mM) or high (25 mM) glucose concentration, cellular proliferation was much more rapid. After 6 h, cells incubated with a physiologic or high glucose concentration demonstrated steady growth, which persisted for 48 h. The viability of these cells also remained quite high at 90.1 ± 1.5% (n = 4) and 94.3 ± 2.0% (n = 4) in the 5 and 25 mM glucose groups, respectively.

Lactate Production in Medium. Lactate production was assessed to determine the effect of glucose concentration on the rate of glycolysis. Lactate measurements were performed after various times of growth in low, physiologic, and high glucose concentrations. MCF-7 cells grown at a low glucose concentration (n = 3) demonstrated no detectable production of lactate from 4 to 24 h, and only a small amount at 48 h (49.4 ± 7.2 μM) (Figure 2). This low level of lactate production was abrogated when cells were grown in low-glucose medium without glutamine (data not shown). Levels of lactate in cells incubated with a physiologic or high glucose concentration (n = 3) increased at an almost linear rate, with relatively large increases (207.6 ± 21.9 and 189.1 ± 24.2 μM, respectively) at 12 h ($p < 0.05$). This rate of increase continued with subsequent levels of 451.5 ± 36.8 and 407.4 ± 15.0 μM at 24 h and 855.8 ± 50.5 and 809.5 ± 29.6 μM at 48 h, respectively. These results indicate that very little glycolysis occurs in cells grown at a low glucose concentration and that glutamine is utilized as a primary energy source in these cells. On the other hand, a progressive increase in the glycolytic rate occurred from 12 to 48 h in cells grown in 5 or 25 mM glucose.

MBP-1 Expression. MBP-1 downregulates c-myc expression and represents a potential mechanism for limiting cell growth under conditions in which energy supplies are limiting (low glucose concentrations). MBP-1 protein expression was assessed to determine if the changes that we observed in cell proliferation reflected changes in MBP-1

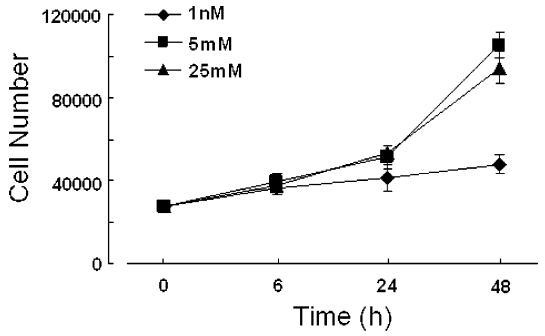

FIGURE 1: Effect of glucose concentration on cell proliferation. Changes in cell proliferation in response to 1 nM (◆), 5 mM (■), or 25 mM glucose (▲) by trypan blue counting of cells. MCF-7 cells were plated (7.5×10^4 per well), and cell counts and viability were measured at 0, 6, 24, and 48 h. Data are means ± sem of three separate experiments.

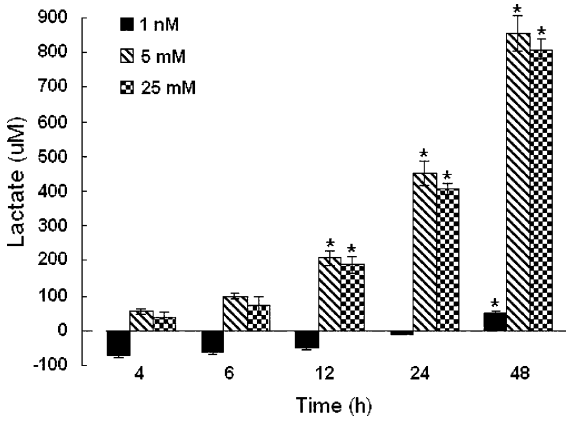

FIGURE 2: Lactate (micromolar) production in medium of MCF-7 cells growing in 1 nM (black bars), 5 mM (striped bars), or 25 mM glucose (checkered bars). Medium was collected at 0, 4, 6, 12, 24, and 48 h, and the lactate concentration was determined by detection of NAD-linked conversion of lactate to pyruvate by lactate dehydrogenase at 340 nm. Values are means ± sem from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in the lactate concentration.

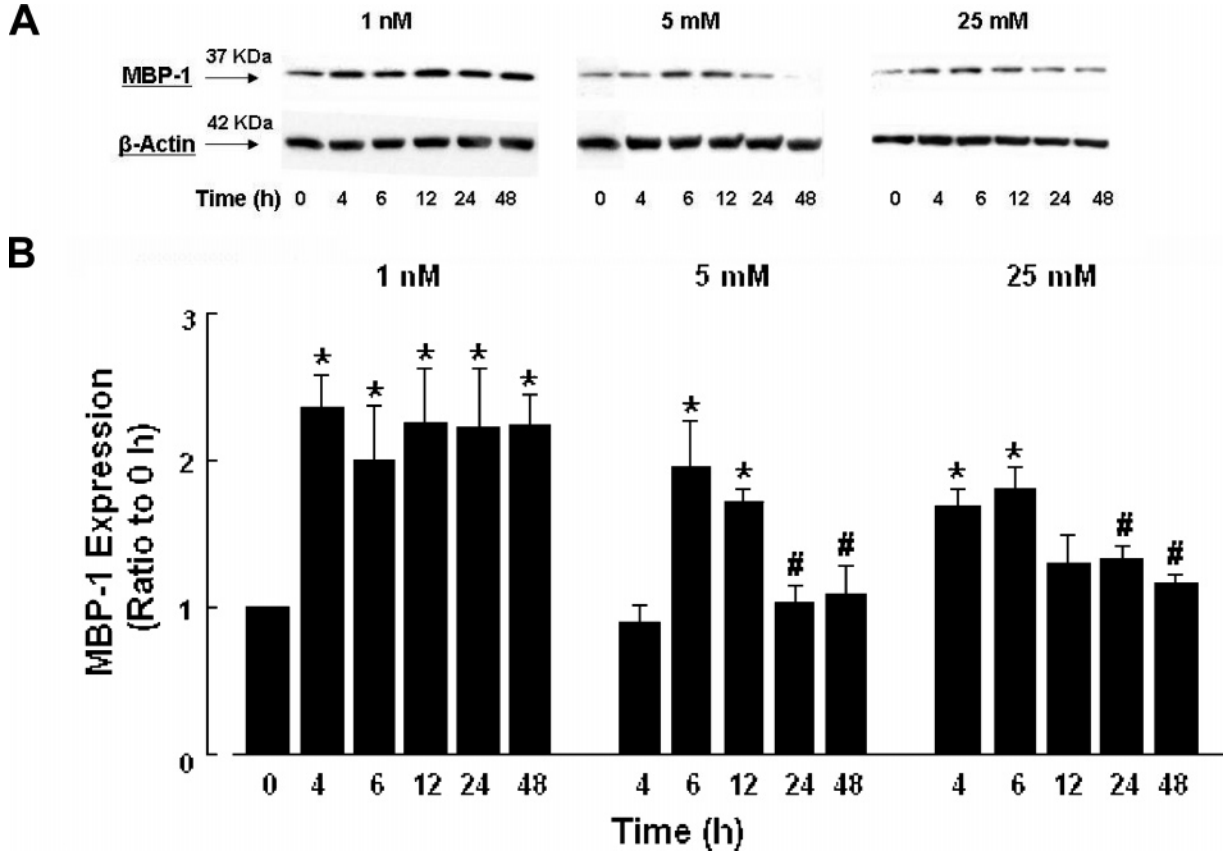

FIGURE 3: Changes in MBP-1 expression with time in response to 1 nM, 5 mM, or 25 mM glucose. (A) Western blots of MBP-1 and β-actin (loading control) from total cell lysates. (B) Histogram of MBP-1 expression at 4, 6, 12, 24, and 48 h. Bars represent means ± sem expressed as a ratio to the 0 h control from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in the level of expression, and number signs denote a significant decrease. Note greater and sustained elevation of MBP-1 with 1 nM glucose and the late (24−48 h) decrease in MBP-1 with 5 and 25 mM glucose.

expression. In fact, incubation of cells in 1 nM glucose caused a sustained increase ($p < 0.05$) in the level of MBP-1 expression at all time points compared to the 0 h control (Figure 3). Exposure of cells to 5 mM glucose significantly enhanced MBP-1 expression at 6 and 12 h (2.03 ± 0.31 and 1.78 ± 0.089, respectively) compared to 0 and 4 h (0.93 ± 0.13); however, the level of expression declined ($p < 0.05$) at 24 (1.05 ± 0.12) and 48 h (1.13 ± 0.19). In the 25 mM glucose group, the level of MBP-1 was elevated ($p < 0.05$) at 4 h (1.64 ± 0.11) and 6 h (1.76 ± 0.14) compared to that at 0 h and was significantly decreased at 24 h (1.30 ± 0.08) and 48 h (1.16 ± 0.05). On average, a lower glucose concentration caused a greater, more sustained increase in the level of MBP-1 expression. This significant and persistent increase in the level of MBP-1 expression, particularly in the cells exposed to low glucose concentrations, may be responsible for the marked decrease in the rate of cellular proliferation.

α-*Enolase Expression and Activity.* Since enolase activity is a marker of glycolysis, changes in α-enolase protein expression and transcript levels were measured using Western blot analysis and RT-PCR, respectively. α-Enolase protein levels were increased by 3-fold at 4 h in response to a low glucose concentration (2.69 ± 0.35 compared to that at 0 h) (Figure 4) as measured by Western blot analysis. However, this initial elevation was followed by a decline ($p < 0.05$) at 6, 12, and 24 h, reaching a level of 0.61 ± 0.06 of 0 time at 48 h. With a physiologic glucose concentration, α-enolase protein levels also increased at 4 h (albeit to a much lesser extent than with low glucose) and were significantly greater than the 0 h control at 6 h (1.64 ± 0.11). This initial increase was followed by a decline at 24 h (0.70 ± 0.10) and 48 h (0.71 ± 0.08). Incubation of cells in high (25 mM) glucose concentrations did not result in an increase in the level of α-enolase expression above control at 4 h (1.02 ± 0.05); however, the level of expression was decreased ($p < 0.05$) at 6 and 12 h and continued to decline at 24 h (0.37 ± 0.11) and 48 h (0.43 ± 0.08). These results demonstrate that lower concentrations of glucose initially induce greater and more prolonged α-enolase protein expression.

α-Enolase transcript levels were significantly decreased at 4 and 6 h compared to that at 0 h in response to 1 nM glucose [$p < 0.05$ ($n = 3$)] (Figure 5). The level of expression had returned to near-control levels by 24 h and was somewhat increased (compared to that at 0 h) by 48 h (1.28 ± 0.14). Exposure of cells to 5 mM glucose ($n = 3$) also resulted in a decreased ($p < 0.05$) level of α-enolase mRNA from 4 to 6 h compared to that at 0 h; however, transcript concentration returned to control levels at 12 h (0.92 ± 0.06), 24 h (1.02 ± 0.14), and 48 h (0.90 ± 0.06). Although the increase in the level of α-enolase mRNA occurred earlier with 5 mM glucose compared to that with 1 nM glucose, the increase at 48 h on average was greater with 1 nM glucose. With 25 mM glucose ($n = 3$), similar to the 5 mM glucose group, the level of α-enolase mRNA was decreased from 4 to 6 h compared to that at 0 h and rose ($p < 0.05$) above the earlier time points to control levels at 12, 24, and 48 h (0.84 ± 0.05, 0.93 ± 0.02, and 1.19 ± 0.19,

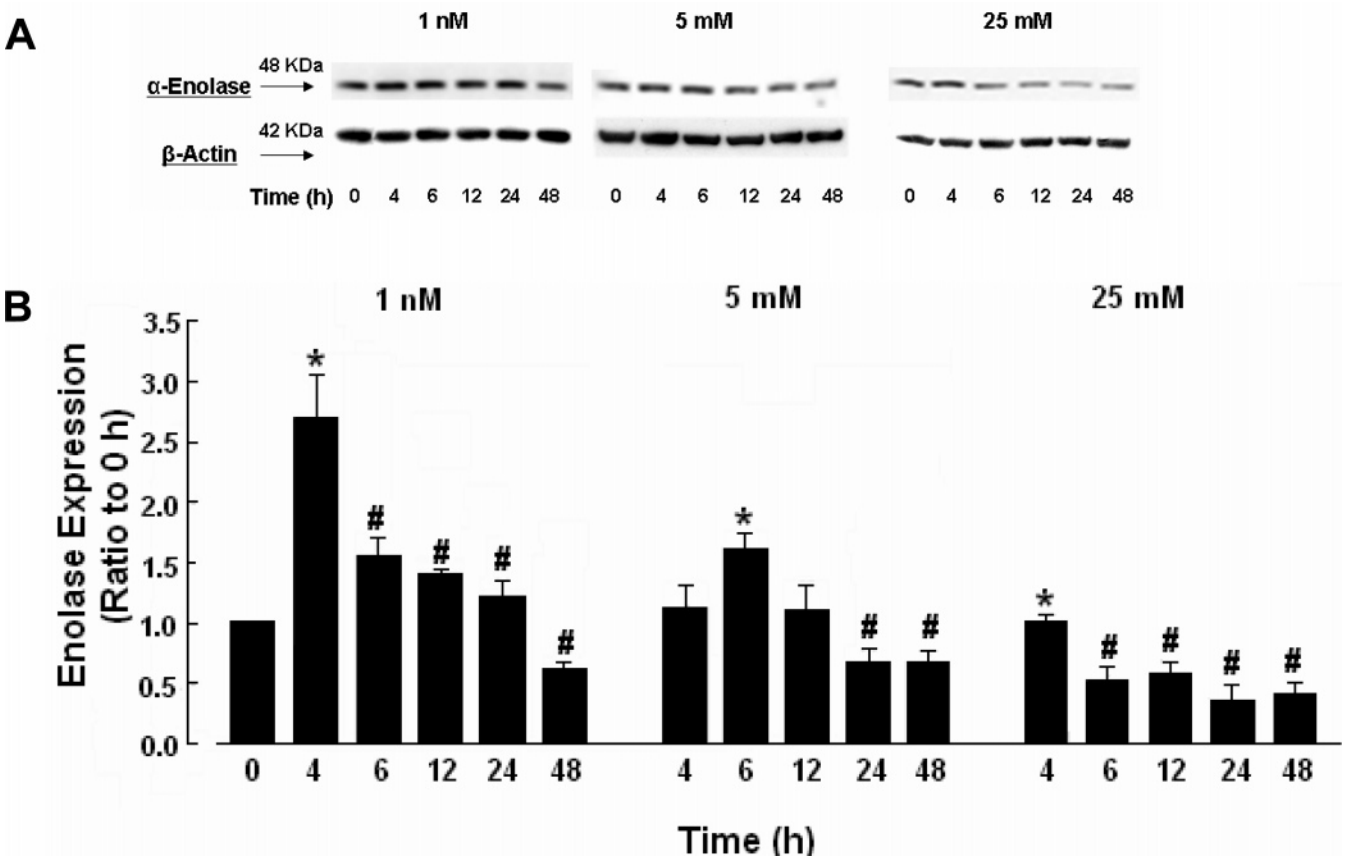

FIGURE 4: Changes in α-enolase expression with time in response to 1 nM, 5 mM, or 25 mM glucose. (A) Western blots of α-enolase and *β*-actin (loading control) from total cell lysates. (B) Histogram of α-enolase expression at 4, 6, 12, 24, and 48 h. Bars represent means ± sem expressed as a ratio to the 0 h control from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in the level of expression, and number signs denote a significant decrease. Note the prominent increase in α-enolase at 4 h with 1 nM glucose, significantly greater than that with 5 and 25 mM glucose.

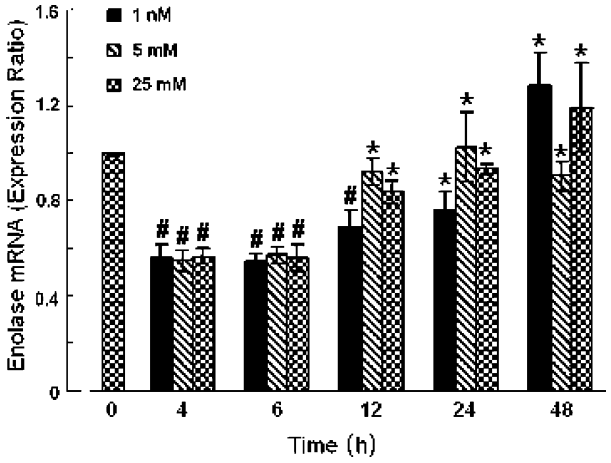

FIGURE 5: Effect of altered glucose concentration on α-enolase mRNA expression measured by RT-PCR. α-Enolase mRNA was compared in MCF-7 cells incubated in 1 nM (black bars), 5 mM (striped bars), or 25 mM glucose (checkered bars) at 4, 6, 12, 24, and 48 h. All measurements were standardized with *β*-actin (loading control) and expressed as a ratio to the 0 h control (expression ratio). Bars represent means ± sem from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in the level of mRNA compared to the values at 4 and 6 h, and number signs denote a significant decrease compared to the 0 h control. Note the increase in α-enolase mRNA from 24 to 48 h with the 1 nM glucose group and the increase from 12 to 48 h with 5 and 25 mM glucose.

respectively). Therefore, a low glucose concentration induced greater α-enolase mRNA levels at 48 h. The increase in the level of α-enolase protein expression at 4 and 6 h with 1 nM and 5 mM glucose, respectively, was not associated with an increase in α-enolase transcript levels.

To determine if the changes in enolase protein concentration paralleled enolase enzymatic activity, enzyme activity was measured in total cell lysates. Cells grown with a low glucose concentration demonstrated a decrease ($p < 0.05$) in enolase activity at 12 h (58.3 ± 13.6% of the 0 h value) (Figure 6). The level of enolase activity did not return to the control level during the course of the experiment, remaining at 74.5 ± 4.0% of the 0 h value after low-glucose concentration exposure for 48 h. With a physiologic glucose concentration, the initial decrease in enolase activity was less pronounced, but was followed by a gradual decrease to 69.4 ± 5.5% of 0 h values at 48 h. In contrast, cells grown at a high glucose concentration did not demonstrate the immediate decrease in enolase activity but did experience a modest decrease at 48 h (77.9 ± 6.8%). These results demonstrate that enolase activity decreases with time in all groups, in parallel to the level of enolase protein expression.

c-myc Expression. c-myc expression is generally a reliable marker of cellular proliferation. It has an interesting regulatory relationship to the α-enolase gene in that its expression is negatively regulated by MBP-1 and it induces α-enolase transcription. Cells grown in a low concentration of glucose demonstrated a robust elevation in the level of c-myc protein expression ($p < 0.05$) at 4 h (6.38 ± 1.05) compared to that at 0 h (Figure 7). This was followed by a relatively rapid

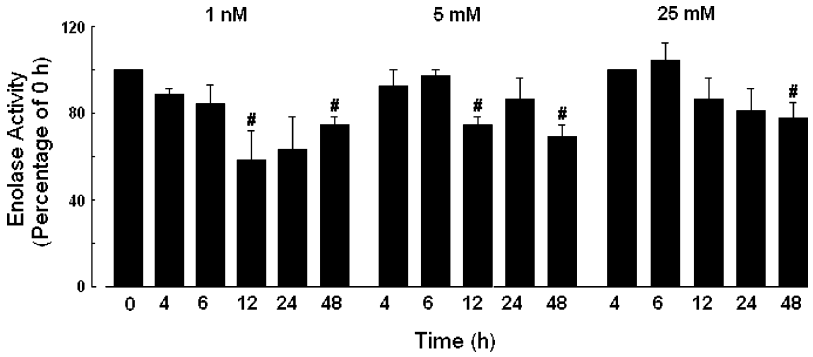

FIGURE 6: Effect of glucose concentration on enolase activity. Conversion of the substrate was assessed at 240 nm, and activity was assessed by changes in V_{max}. Bars represent means ± sem of three separate experiments expressed as a percentage of the 0 h control. Number signs denote a significant ($p < 0.05$) decrease in activity compared to that at 4 h.

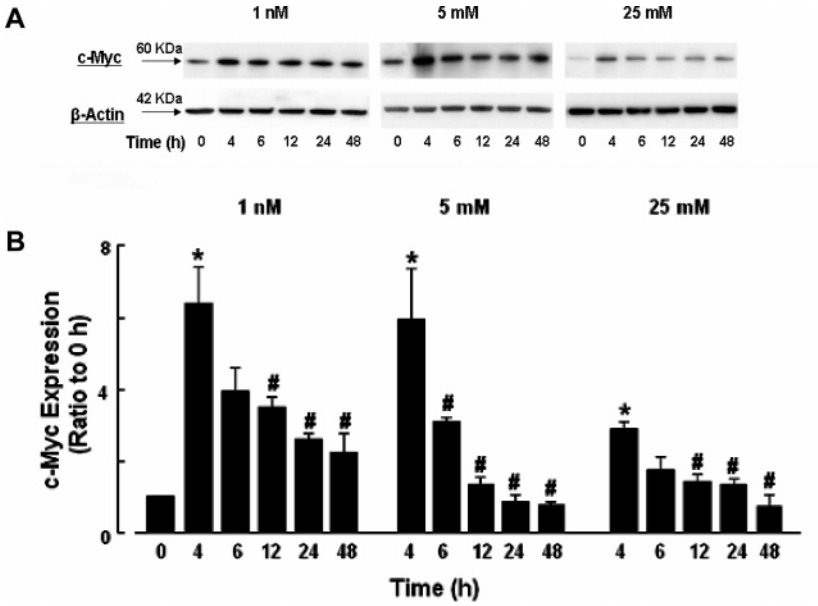

FIGURE 7: Time course of c-myc expression in response to 1 nM, 5 mM, or 25 mM glucose. (A) Western blots of c-myc and β-actin (loading control) from total cell lysates. (B) Histogram of c-myc expression at 4, 6, 12, 24, and 48 h. Bars represent means ± sem expressed as a ratio to the 0 h control from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in the level of expression, and number signs denote a significant decrease. Note the increase in the level of c-myc expression at 4 h, which is greater in the 1 nM and 5 mM groups than in the 25 mM group.

decline, although c-myc levels did not reach 0 h levels in cells grown at the low glucose concentration. Cells grown at the physiologic glucose concentration also demonstrated a dramatic increase in the level of c-myc expression ($p < 0.05$) at 4 h (5.96 ± 1.40) compared to 0 h followed by a gradual decrease in protein levels at 6 h (3.14 ± 0.11), 12 h (1.41 ± 0.14), 24 h (0.89 ± 0.19), and 48 h (0.82 ± 0.082). Cells grown in 25 mM glucose had a highly attenuated early c-myc response, although levels increased to 2.88 ± 0.21 at 4 h ($p < 0.05$) compared to that at 0 h. Expression steadily declined ($p < 0.05$) at 12 h (1.44 ± 0.16), 24 h (1.36 ± 0.15), and 48 h (0.80 ± 0.26) compared to that at 4 h. These results indicate that c-myc protein expression is upregulated early as part of an "early cellular response" to low levels of glucose. This response may be an important element of the cellular metabolic reaction to low glucose concentrations.

Effect of Glucose Concentration on c-myc and glut-1 mRNA Expression. Changes in c-myc and glut-1 transcript levels were measured with RT-PCR. In cells grown in 1 nM glucose ($n = 3$), there was no early increase in c-myc mRNA, which could account for the increase in c-myc protein at 4 h documented in Figure 7. However, the level of c-myc transcripts was significantly ($p < 0.05$) decreased at 6 h (0.87 ± 0.04) and 12 h (0.76 ± 0.06) compared to that at 0 h (Figure 8). At 24 h of low glucose exposure, cells began exhibiting increased levels of c-myc mRNA, reaching an almost 3.5-fold increase by 48 h. The dramatic increase in c-myc mRNA was not associated with an increase in c-myc protein expression. On the other hand, with cells grown at physiologic and high glucose concentrations, the level of c-myc mRNA did not increase at 4 h and was significantly decreased at 6, 12, and 24 h compared to that at 0 h. In contrast to the low-glucose concentration group, levels of c-myc mRNA returned to control levels at 48 h and were not significantly increased compared to that at 0 h. These results indicate that the increment in the level of c-myc protein at 4 h must be regulated at the post-transcriptional level. The dramatic increase in the level of c-myc transcripts at 48 h with 1 nM glucose may reflect a response to metabolic stress.

Concurrent with changes in c-myc transcripts, the level of glut-1 mRNA was elevated in the low-glucose group ($p < 0.05$) at 24 h (1.46 ± 0.10) and 48 h (2.29 ± 0.18) compared to all time points (Figure 9). Cells incubated in

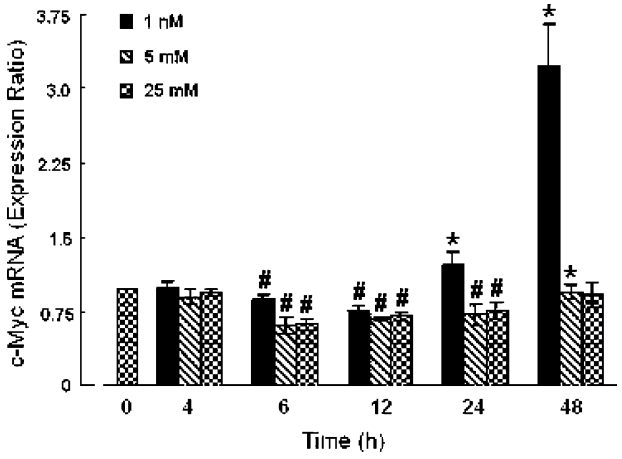

FIGURE 8: Effect of altered glucose concentration on c-myc mRNA expression measured by RT-PCR. c-myc mRNA was compared in MCF-7 cells incubated in 1 nM (black bars), 5 mM (striped bars), or 25 mM glucose (checkered bars) at 4, 6, 12, 24, and 48 h. All measurements were standardized with β-actin (loading control) and expressed as a ratio to the 0 h control (expression ratio). Bars represent means ± sem from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in mRNA, and number signs denote a significant decrease. Note the pronounced increase in c-myc transcription at 48 h with 1 nM glucose and a return in c-myc to control levels with 5 and 25 mM glucose.

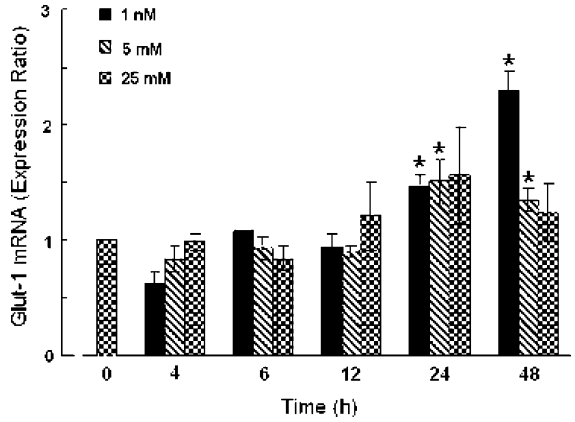

FIGURE 9: Time course of glut-1 mRNA in response to an altered glucose concentration measured by RT-PCR. glut-1 mRNA was compared in MCF-7 cells incubated in 1 nM (black bars), 5 mM (striped bars), or 25 mM glucose (checkered bars) at 4, 6, 12, 24, and 48 h. All measurements were standardized with β-actin (loading control) and expressed as a ratio to the 0 h control (expression ratio). Bars represent means ± sem from three separate experiments. Asterisks denote a significant ($p < 0.05$) increase in mRNA. Note the pronounced increase in glut-1 mRNA from 24 to 48 h with 1 nM and 5 mM glucose. High glucose did not increase glut-1 mRNA.

physiologic and high glucose concentrations demonstrated a more modest increase in glut-1 mRNA. Incubation of cells in 5 mM glucose ($n = 3$) also significantly increased glut-1 mRNA at 24 h (1.51 ± 0.19) compared to 0, 4, and 12 h and at 48 h (1.35 ± 0.10) compared to all time points. However, the increase at 48 h was greater ($p < 0.05$) with 1 nM glucose than with 5 mM glucose. No significant changes in glut-1 mRNA were measured in the 25 mM glucose group ($n = 3$).

Loss of MBP-1 Binding to the P_2 Promoter at 48 h in Response to 1 nM Glucose. MBP-1 has been shown to bind to a DNA sequence containing the TATA box of the c-myc P_2 promoter and downregulate c-myc transcription. Since a low glucose concentration induces an increase in c-myc

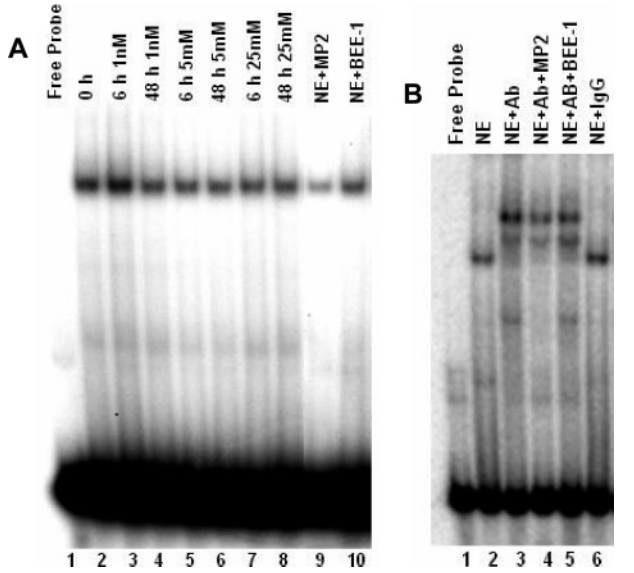

FIGURE 10: Altered P_2 promoter binding of c-myc promoter binding protein in response to changes in glucose concentration as determined by an electrophoretic mobility shift assay. (A) A 50 bp c-myc P_2 promoter was labeled with ^{32}P and incubated with nuclear extract (NE). Cold competitor MP2 (150 nM) (lane 9) or cold nonspecific oligonucleotide BEE-1 (150 nM) (lane 10) was added prior to addition of the probe. Note the prominent binding of MBP-1 to the probe at 6 h with 1 nM glucose (lane 3) and a decreased level of binding at 48 h (lane 4). No changes in MBP-1 promoter binding occurred between 6 and 48 h in the 5 mM (lanes 5 and 6) or 25 mM (lanes 7 and 8) glucose groups; however, the overall level of binding was decreased. (B) An α-enolase antibody was added to a reaction mixture containing the nuclear extract and ^{32}P-labeled P_2 promoter (lane 3), causing a supershift of the DNA−protein complex. This supershift was challenged with MP2 cold competitor (lane 4), but not with the cold nonspecific competitor BEE-1 (lane 5). No supershift occurred with addition of rabbit IgG to the reaction mixture in place of antibody (lane 6).

mRNA concentration at 48 h in conjunction with an increase in the level of MBP-1 expression, inconsistent with the role of MBP-1 as a negative regulator of c-myc transcription, we were interested in whether a change in MBP-1 P_2 promoter binding activity occurred. We addressed this question via an EMSA using a 50 bp labeled oligonucleotide relative to the P_2 start site on the c-myc promoter (Figure 10A). A DNA−protein complex was visualized by autoradiography, and its specificity was confirmed when the complex was disrupted with a 150 nM excess of cold MP2 competitor, but not with a 150 nM excess of the cold nonspecific competitor BEE-1. Nuclear extracts from cells grown in 1 nM glucose showed a prominent increase in the level of MBP-1 promoter binding at 6 h relative to 0 h, which was 3-fold higher than that for the 6 h samples from the 5 and 25 mM glucose groups. Interestingly, after 48 h with a low glucose concentration, MBP-1 promoter binding activity decreased by 3-fold, co-incident with the prominent increase in the level of c-myc transcription. There was no change in MBP-1 promoter binding between 6 and 48 h in the 5 and 25 mM glucose groups; however, overall levels of promoter binding were decreased compared to that at 0 h. An α-enolase polyclonal antibody was able to bind and supershift the DNA−protein complex and could be effectively challenged with excess MP2 but not BEE-1, further demonstrating specificity of the shifted complex (Figure 10B).

DISCUSSION

We have previously demonstrated that MBP-1, an alternative translation initiation product of the α-enolase gene, negatively regulates c-myc expression and cellular proliferation (*16*). It is not surprising, given its ability to downregulate c-myc expression, that MBP-1 functions as a tumor suppressor (*12*). On the other hand, c-myc overexpression stimulates expression of several glycolytic pathway enzyme genes, including α-enolase. Thus, the ability of an enolase gene product (MBP-1) to downregulate c-myc expression provides a potentially important "feedback loop" for regulating c-myc expression in response to factors altering the activity of the glycolytic pathway. This may provide a mechanism by which cells can alter their proliferative rate in response to changes in exogenous nutrient concentrations.

In this study, we characterize the role of α-enolase and MBP-1 in the changes in cell proliferation induced by an altered glucose concentration. The data presented here support the hypothesis that translational regulation of MBP-1 expression and post-translational modification of its function are important factors in regulating the cellular response to glucose deprivation. To our knowledge, this is the first description of changes in endogenous MBP-1 and its effect on mediating growth arrest. Our results suggest that enolase and MBP-1 provide an important regulatory convergence between cell growth and energy metabolism.

Low glucose concentrations induced a striking increase in the level of MBP-1 expression, which was accompanied by markedly inhibited cell growth. Interestingly, these changes were not accompanied by corresponding changes in α-enolase mRNA. In fact, α-enolase mRNA levels decreased during the period during which MBP-1 protein levels were elevated. This suggests that the changes induced by a low glucose concentration are mediated through preferential translation of enolase mRNA using the downstream (MBP-1) translation initiation start site.

In contrast, cells grown at physiologic and high glucose concentrations demonstrated a steady increase in the rate of proliferation from 12 to 48 h, with a corresponding decrease in the level of MBP-1 expression. At the same time, lactate production in the medium became markedly elevated, reflecting the hyperglycolytic phenotype of these cells indicating an association between loss of MBP-1 and growth stimulation. The striking difference in lactate production between cells grown at low and high glucose concentrations demonstrates the necessity of adequate glucose levels for increased glycolytic flux.

The initial increase in the level of MBP-1 expression at low, physiologic, and high glucose concentrations resulted in a concomitant decrease in the level of c-myc transcription, consistent with the role of MBP-1 as a negative regulator of c-myc transcription. This is similar to previous reports which showed c-myc mRNA suppression by transfection of MCF-7 cells with exogenous MBP-1 (*10*). However, at the physiologic and high glucose concentrations, the subsequent decrease in the level of MBP-1 expression corresponded with a return in c-myc mRNA to control levels. Interestingly, although the low-glucose group exhibited a sustained high level of MBP-1 expression, the rate of c-myc transcription increased dramatically at 48 h, an increase not seen in the physiologic- and high-glucose conditions. Previous work by other groups also demonstrated a 3−4-fold increase in the level of c-myc mRNA in cells cultured under low-glucose conditions compared to higher glucose concentrations (*17*). This is consistent with the role of c-myc as an "early response" gene whose expression is stimulated by a variety of stress types (*18*).

The increase in c-myc transcription at 48 h in response to a low glucose concentration, despite persistently elevated MBP-1 expression, suggested a possible loss of MBP-1 repression of transcription. Indeed, the demonstration via an EMSA of decreased MBP-1 binding to the c-myc P_2 promoter at 48 h supports this hypothesis. While extracts from cells grown in low glucose demonstrated considerable MBP-1 binding to the c-myc P_2 promoter at 6 h, this binding declined by 3-fold at 48 h, in concordance with increased c-myc transcription. The loss of promoter binding was not seen in extracts from cells grown in physiologic or high glucose. In fact, overall levels of promoter binding were decreased at 6 and 48 h with 5 and 25 mM glucose, ideal for growth stimulation. Previously, the 16 kDa protein, MBP-1 interacting protein-2A (MIP-2A), has been shown to interact with MBP-1 in vivo and in vitro and antagonize MBP-1-mediated transcriptional repression of the c-myc promoter (*19*). Therefore, increased binding of MBP-1 to MIP-2A in response to low glucose may well account for the decrease in c-myc P_2 promoter binding at 48 h; however, this remains to be investigated.

Since persistent expression of MBP-1 may induce cell death as shown with human fibroblasts (*20*) and carcinoma (*21*), inhibition of its function may be an adaptation which allows transformed cells to avoid apoptosis, which may occur with continual repression of c-myc transcription. Thus, the abrogation of MBP-1 activity might provide a theoretical advantage by allowing cancer cells to survive under limited nutrient availability. The significant changes in binding of MBP-1 to the c-myc P_2 promoter at low glucose concentrations strongly support an important role for MBP-1 in this adaptive response.

The effect of low glucose on c-myc protein expression does not appear to be directly modulated through MBP-1 levels. Although c-myc expression was elevated at 4 h at all glucose concentrations, low glucose induced a 2-fold greater level of c-myc expression than high glucose in the presence of higher levels of MBP-1. The discrepancy between c-myc mRNA and protein levels may be due to the ability of c-myc protein to be modified post-translationally such as by phosphorylation, which regulates its destruction by ubiquitination (22). Since c-myc plays an important role in both cell growth and apoptosis, it is important that c-myc expression be tightly regulated by several control mechanisms. Other studies support our findings demonstrating that glucose deprivation (*23*, *24*) increases c-myc expression, which may increase the accumulation and utilization of glucose (*25*, *26*). Several mechanisms have been suggested for the increase in c-myc in response to low glucose, including a reduction in mitochondrial metabolism (*27*) as a result of accumulation of reactive oxygen species and ATP depletion (*28*).

The level of α-enolase protein expression did not appear to be directly related to α-enolase mRNA. There was an initial increase in α-enolase expression which peaked

between 4 and 6 h at all glucose concentrations and was greater in the 1 nM and 5 mM glucose groups than in the 25 mM glucose group. From 6 to 48 h, α-enolase expression decreased in all groups; however, the rate of the decline was slower with 1 nM glucose compared to 5 mM glucose. In parallel with α-enolase expression, activity also decreased with time. The change in α-enolase expression directly corresponded with the rise and fall in c-myc expression, suggesting regulation of α-enolase by c-myc.

Since the promoter of the α-enolase gene has two c-myc binding motifs, c-myc can directly transactivate α-enolase, leading to an increase in glucose uptake (*5*, *8*). We found that 1 nM and 5 mM glucose induce greater c-myc expression initially, which therefore may account for higher levels of α-enolase at 4 h. Studies suggest that induction of α-enolase in response to oxidative stress may function to maintain ATP levels (*29*). Therefore, induction of α-enolase in response to low glucose may help to compensate for a reduction in the cellular metabolism. A decrease in α-enolase expression in the cytosol and nucleus was also reported in non-small cell lung carcinoma, suggesting downregulation of both α-enolase and MBP-1. Tumors with α-enolase downregulation also tended to be larger, suggesting an association between loss of MBP-1 and tumor progression (*13*).

Although α-enolase is an important player in the glycolytic pathway, protein expression of α-enolase actually decreased over time as levels of lactate increased at all glucose concentrations. Key enzymes such as phosphofructokinase, pyruvate kinase, hexokinase II, and enolase are subject to allosteric regulation by ATP. When the rate of glycolysis increases, resulting in an elevation of the level of intracellular ATP, enolase expression and activity may correspondingly be decreased (*30*).

The glucose transporter, glut-1, is directly transactivated by c-myc in the control of glycolytic flux (*5*). Its overexpression correlates with poor prognosis and tumor aggressiveness in cancer patients (*31*, *32*). Our results show an increase in glut-1 mRNA at 48 h in response to a low glucose concentration, consistent with the increase in glycolysis as reflected in increased lactate production. Although a modest increase in glut-1 mRNA was measured at 48 h in physiologic glucose, the magnitude of the increase was much smaller than that seen in low glucose. No significant increase in glut-1 was measured in high glucose, suggesting that expression of the glut-1 transporter is dependent on glucose availability. Previous studies in esophageal, gastric, breast, and colon carcinomas also suggest that cellular competition for increasingly limited resources will favor phenotypes with increased expression of glut-1 (*33*). This may be a critical factor in preventing the induction of apoptosis in cells by limited nutrient supply (*23*).

In summary, this study shows that MBP-1 plays a pivotal role in regulating proliferation of MCF-7 cells in response to an altered glucose concentration. These results suggest that α-enolase and MBP-1 may play a central role in both glucose metabolism and growth regulation of transformed cells and may be a likely candidate for the development of clinical therapies. This provides an important intersection of the metabolic and growth regulatory roles of this important gene.

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