Glucose Catabolism in Cancer Cells

IDENTIFICATION AND CHARACTERIZATION OF A MARKED ACTIVATION RESPONSE OF THE TYPE II HEXOKINASE GENE TO HYPOXIC CONDITIONS

Saroj P. Mathupala, Annette Rempel, and Peter L. Pedersen

From the Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205-2185

One of the most common signatures of highly malignant tumors is their capacity to metabolize more glucose to lactic acid than their tissues of origin. Hepatomas exhibiting this phenotype are dependent on the high expression of type II hexokinase, which supplies such tumors with abundant amounts of glucose 6-phosphate, a significant carbon and energy source especially under hypoxic conditions. Here we report that the distal region of the hepatoma type II hexokinase promoter displays consensus motifs for hypoxia-inducible factor (HIF-1) that overlap E-box sequences known to be related in other gene promoters to glucose response. Moreover, we show that subjecting transfected hepatoma cells to hypoxic conditions activates the type II hexokinase promoter almost 3-fold, a value that approaches 7-fold in the presence of glucose. Consistent with these findings is the induction under hypoxic conditions of the HIF-1 protein. Reporter gene analyses with a series of nested deletion mutants of the hepatoma type II hexokinase promoter show that a significant fraction of the total activation observed under hypoxic conditions localizes to the distal region where the overlapping HIF-1/E-box sequences are located. Finally, DNase I footprint analysis with a segment of the promoter containing these elements reveals the binding of several nuclear proteins. In summary, these novel studies identify and characterize a marked glucose-modulated activation response of the type II hexokinase gene to hypoxic conditions within highly glycolytic hepatoma cells, a property that may help assure that such cells exhibit a growth and survival advantage over their parental cells of origin.

Numerous studies have demonstrated that highly malignant tumors, i.e. those that are poorly differentiated and grow rapidly, exhibit the capacity to metabolize glucose to lactic acid at much higher rates than normal cells (reviewed in Refs. 1–5).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U19605.

The abbreviations used are: HIF-1, hypoxia-inducible factor-1; p.s.i., pounds per square inch; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid.
With the above thoughts and information serving as a guide, we proceeded to identify HIF-1 binding sites on the hepatoma type II hexokinase promoter and to characterize in some detail the promoter’s capacity to be regulated under hypoxic conditions. As predicted above and described in detail below, hypoxic stress significantly activates the promoter, a response that is further accentuated by physiological concentrations of glucose and correlates with the induction of HIF-1 expression.

**EXPERIMENTAL PROCEDURES**

**Materials**

The pGL2-Basic luciferase reporter vector, the pSvβ-β-galactosidase control vector, and the luciferase assay system were from Promega. The TD-20e luminometer for chemiluminescence measurements and the portable anaerobic chamber were purchased from Turner Designs and Fisher, respectively. The cell porator (electroporator) for transfecting mammalian cells, restriction enzymes, DNA modifying enzymes, and an antibiotic-antimyotic mixture were purchased from Life Technologies, Inc. The glucose-deficient RPMI 1640 tissue culture medium was from Sigma. The Maxi Prep plasmid purification system used to prepare plasmid DNA for transfections was from Qiagen, and the monoclonal antibody against the α subunit of HIF-1 (HIF-1α) was from Novus Biologicals. Polyvinylidene difluoride membranes were from Bio-Rad, and the ECL chemiluminescence system for detecting the antibody was from Amersham Pharmacia Biotech. [α-32P]dATP (3000 Ci/mmol) and [α-35S]dATP (1000 Ci/mmol) were from PerkinElmer Life Sciences. A gas mixture of 1% O₂, 5% CO₂, and 94% N₂ was obtained from MG Industries. A proximal promoter of the type II hexokinase gene derived from AS-30D hepatoma cells was isolated, sequenced, and characterized as described in earlier reports from this laboratory (10–13). Female Sprague-Dawley rats were obtained from Charles River Laboratories. Their care and use in the experiments described below was approved by and conducted in accordance with the guidelines of the Johns Hopkins University Animal Care and Use Committee. The AS-30D cells, an established mouse cell line exhibiting a high glycolytic rate (6, 7), is routinely maintained and propagated in the laboratory of the corresponding author.

**Methods**

**Tumor Cells—**AS-30D hepatoma cells were propagated in the peri-toneal cavity of female Sprague-Dawley rats (100–150 g) and isolated as described previously using glucose-supplemented RPMI 1640 medium with 10% fetal bovine serum (8). They were then resuspended at a final concentration of 50 × 10⁶ cells ml⁻¹ for transfient transfection studies (10).

**Detection of HIF-1—**To assess the levels of HIF-1 in AS-30D hepatoma cells, the cells were washed once in Dulbecco’s modified phos-phate-buffered saline (Ca²⁺-, Mg²⁺-free). Nuclear extracts were prepared from the washed cells as described below, and analyzed by SDS-PAGE and Western blotting using HIF-1 antibody from Novus Biologicals, the reliability of which has been documented in recent studies (20, 21).

**Western Analysis—**For Western blotting, samples of nuclear extracts from AS-30D hepatoma cells were subjected to SDS-PAGE on 7.5% gels, which were then transferred onto polyvinylidene difluoride membranes in CAPS buffer (10 mM CAPS, 10% v/v methanol, pH 11). The membranes were probed with an anti-HIF-1α monoclonal antibody, according to the manufacturer’s instructions, and detected by an ECL chemiluminescence system.

**Preparation of Nuclear Extracts—**Nuclear extracts were prepared according to the method of Dignam et al. (22) from nuclei isolated from fresh AS-30D hepatoma cells and then stored at −80 °C until use.

**Construction of Nested Deletion Mutants for Reporter Gene Analyses—**The preparation of the hepatoma type II hexokinase promoter-luciferase construct containing the full-length 4.3-kbp promoter for transfection conditions and subsequent characterization of the full-length hexokinase promoter-luciferase construct and the β-galactosidase control vector have been reported previously (10). The same transfection conditions were used in this study for these constructs and also for the constructs containing the nested deletion mutants. Briefly, equimolar concentrations of reporter gene vector DNA (equivalent to 10 µg) of the 4.3-kbp full-length promoter-luciferase reporter gene construct were maintained during transfections with the nested deletion mutant constructs (i.e., 8.7, 7.8, 6.8, 5.9, and 5.7 µg, respectively, of 3-, 2-, 1-, 0.1-, and 0-kbp promoter containing nested deletion mutant plasmids). The β-galactosidase vector (2.5 µg) was used as a control in all transfections, where 25 × 10⁶ hepatoma cells (in 0.5 ml) were used per transfection. The cells and reporter gene constructs were incubated in 25 ml of DMEM at 20°C for 24 h. After an additional 10-min recovery period on ice, the transfected AS-30D hepatoma cells were plated into 10 ml of RPMI 1640 glucose-deficient medium supplemented with 2 mM glutamine, 1 mM pyruvate, and 25 mM HEPES (pH 7.4), and an antibiotic-antimyotic mixture. For studies in the presence of glucose, the medium was supplemented with 5 mM glucose. For optimal results under normoxic conditions, incubation of cells was carried out at 37°C in 5% CO₂ and 95% atmospheric air for 24 h. The cells were then subjected to lysis using a cold buffer containing 0.6% Triton X-100, 0.1 mM potassium phosphate, and 1 mM DTT, pH 7.8 (25). Finally, aliquots (10 and 50 µl) were used to assay luciferase and β-galactosidase activity, respectively.

**Incubation of Transfected Cells Under Hypoxic Conditions—**To expose transfected cells to hypoxia, parallel sets of 100-mm tissue culture dishes containing 25 × 10⁶ transfected cells/plate were placed in a portable anaerobic chamber. This was sealed and flushed with an “anaerobic” gas mixture of 1% O₂, 5% CO₂, 94% N₂ for 2 min, followed by five successive evacuations (at low vacuum, −1–2 p.s.i.) and repressurization (with the same gas mixture at −1–2 p.s.i.) steps. The chamber was finally flushed again with the anaerobic gas mixture, the medium was aspirated, and transfected cells were incubated for 8 h in a 37°C incubator. For optimal results, cells were recovered 12–16 h after transfection, washed once in 10 ml of Dulbecco’s modified phosphate-buffered saline (Ca²⁺-, Mg²⁺-deficient), and lysed in 100 µl of cold lysis buffer (0.625% Triton X-100, 0.1 mM potassium phosphate, 1 mM DTT, pH 7.8). Aliquots (10 and 50 µl) were then assayed for luciferase and β-galactosidase activities, respectively.

**Luciferase and β-Galactosidase, and Quantification of Data—**These enzyme assays were carried out essentially as described by the suppliers of the reporter gene vectors (Promega). Luciferase activity (A) was measured by luminometry using a Turner TD-20e luminometer and recorded as relative light units. The system contained 10 µl of cell lysate and 100 µl of a mixture containing 470 µg luciferin, 530 µM ATP, 270 µM CoA, 2.67 mM MgSO₄, 1.07 mM (MgCl)₄, 0.02 M Mg(OH)₂, 33 mM DTT, and 0.1 mM EDTA, pH 7.8, 25°C. β-Galactosidase activity (B) was measured using a PerkinElmer Life Sciences Lambda 3B spectrophotometer and recorded as the absorbance at 420 nm. The system contained 50 µl of cell lysate, 100 µl of water, and 150 µM of 2× β-galactosidase assay reagent (1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside, 120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercaptoethanol, pH 7.4). The mixture was incubated at 37°C for 8 h and then quenched on ice, centrifuged, and the supernatant assayed for β-galactosidase activity. Values A/B that normalized the various transfections were then obtained. In these experiments, endogenous β-galactosidase activity, i.e. the activity in nontransfected AS-30D cells, was found in control experiments to be no more than 1/20 that of experimental values obtained for transfected cells. Results are given as the mean values (±SD) of normalized values (A/B) obtained for the 4.3-kbp promoter, or nested deletion mutants thereof (in the presence of glucose alone, hypoxia alone, or glucose + hypoxia) by normalized values obtained using pyruvate-containing post-transfection medium in the absence of these additions (glucose or hypoxic conditions). Each experiment was carried out multiple times and mean values reported.
from methods by Kingston (26) and Christy et al. followed by a 60-s digestion at 25°C of sonicated salmon sperm DNA as competitor DNA, and variable DNA template end-labeled by Klenow fill-in reactions (23, 24), 1000 ng then subjected to autoradiography for 1 h. Results presented in Fig. 1B summarize the results of further analyses of the distal region from −3620 to −3919 kbp for HIF-1 motifs (5′-RCGTC-3′ or 5′-ACGTG-3′). Significantly, both visual inspection and the use of a computer algorithm (Signal-Scan; Ref. 30) revealed the presence of one HIF-1 motif (5′-RCGTC-3′) overlapping the previously identified E-box (CACGTG), and a second HIF-1 motif (5′-RCGTC-3′) about 50 base pairs upstream overlapping a sequence (CACGTG) with 83% identity to the E-box.

**RESULTS**

The Distal Region of the Hepatoma Type II Hexokinase Promoter Displays Two HIF-1 Motifs That Overlap Glucose Response-Related E-Box Sequences (28, 29). In an earlier report (10), we presented the sequence of the 4.3-kbp proximal promoter region of the hepatoma type II hexokinase gene. (GenBank™ accession number U196605), identified an E-box (CACGTG) in the distal region (Fig. 1A), and demonstrated using reporter gene analysis that the promoter undergoes a modest activation response (2–3-fold) in the presence of a high concentration of glucose (25 mM). Results presented in Fig. 1B summarize the results of further analyses of the distal region from −3620 to −3919 kbp for HIF-1 motifs (5′-RCGTC-3′ or 5′-ACGTG-3′). Significantly, both visual inspection and the use of a computer algorithm (Signal-Scan; Ref. 30) revealed the presence of one HIF-1 motif (5′-RCGTC-3′) overlapping the previously identified E-box (CACGTG), and a second HIF-1 motif (5′-RCGTC-3′) about 50 base pairs upstream overlapping a sequence (CACGTG) with 83% identity to the E-box.

**Hypoxic Conditions Activate the Hepatoma Type II Hexokinase Promoter by a Process That Is Enhanced by Physiological Concentrations of Glucose**—The identification of two overlapping E-box/HIF-1 sequences within the distal region of the type II hexokinase promoter raised the possibility that this promoter may be responsive to hypoxic conditions by a process that is modulated by glucose. To test this possibility, AS-30D hepatoma cells freshly isolated from the host animals were transfectioned with a full-length type II hexokinase promoter-luciferase reporter gene construct and exposed in standard medium in separate experiments to three sets of conditions: hypoxia alone (1% O2, 5% CO2, 94% N2), normoxia alone (5 mM glucose, 5% CO2, 95% atmospheric air), and hypoxia plus glucose. As shown in Fig. 1C, hypoxic conditions alone activate the...
promoter almost 3-fold relative to that observed in the presence of a pyruvate containing “background” medium. However, in the presence of hypoxic conditions plus glucose, the latter of which activates the promoter only about 2-fold, the total activation response approaches 7-fold, a value considerably higher than that expected (approximately 5-fold) had the hypoxia + glucose responses been additive. Moreover, this was a reproducible observation in experiments conducted with AS-30D hepatoma cells from different animals. Thus, these results provide evidence that the hepatoma type II hexokinase promoter is activated by hypoxic conditions in hepatoma cells, and that this response is positively modulated in the presence of a concentration of glucose (5 mM) within the physiological range.

The HIF-1 Protein Is Expressed in the Model Hepatoma Cell Line under Study Where Its Level of Expression Can Be Regulated by Oxygen Concentration—The experiments described above showing that the AS-30D hepatoma type II hexokinase promoter is activated by hypoxic conditions in cells freshly isolated from the host animal implicate the presence of the HIF-1 protein. This was confirmed by subjecting nuclear extracts from these cells first to SDS-PAGE (Fig. 2A, left lanes 1 and 2) and then to Western blot analysis using an HIF-1α subunit antibody (Fig. 2A, right lanes 1 and 2). Subsequent experiments carried out by subjecting AS-30D hepatoma cells in tissue culture to normoxic, hypoxic, or hypoxic plus glucose conditions confirmed that HIF-1α subunit levels in these cells are very dependent on oxygen concentration (Fig. 2B). Thus, cells maintained under normoxic conditions expressed much less HIF-1α subunit than cells maintained under hypoxic conditions (Fig. 2B, lower panel, compare lanes 1 and 2 with lanes 3 and 4). Glucose was without effect on the protein expression levels of the HIF-1α subunit under the same conditions (Fig. 2B, lower panel, compare lane 3 with lane 4). In all of these experiments, the apparent molecular mass of the expressed HIF-1α subunit was about 100 kDa; a value that correlates closely to that of the calculated value of 93.3 kDa (GenBank® accession no. Y09507).

Taken together with the experiments described above, these results implicate the HIF-1α subunit as acting within the distal region of the type II hexokinase promoter and participating, at least in part, in the promoter’s activation response to hypoxic conditions. These results also indicate that the role of glucose in enhancing the promoter’s activation response to hypoxic conditions (Fig. 1C) is not the result of an effect on HIF-1α subunit expression.

A Significant Fraction of the Total Hypoxic Response of the Type II Hexokinase Promoter Can Be Functionally Located to the Distal Region Containing the Overlapping E-box/HIF-1 Sequences—A plasmid constructed for luciferase reporter gene analysis using the full-length promoter (Fig. 3A) formed the basis for the deletion analysis study to functionally locate the primary regions within the promoter that are responsive to hypoxia. As described in detail under “Methods,” a series of nested deletion mutants (plasmids B–F) that maintained −1-kbp deletions at the 5’ terminus (Fig. 3B) were selected from the larger pool of deletion mutants generated. During transfection assays with the selected nested deletion mutants, equimolar concentrations of reporter gene vectors equivalent to 10 μg of the 4.3-kbp full-length promoter-luciferase reporter gene construct (plasmid A) were maintained together with β-galactosidase control vector at concentrations indicated under “Methods.” Analysis of these clones by reporter gene analysis under these conditions enabled us to identify the distal 1 kbp of the hepatoma type II hexokinase promoter as a significant contributor to its response to hypoxic conditions in the presence of a physiological concentration (5 mM) of glucose (Fig. 3C). This region of the promoter also contributes in part to the total glucose response. In both cases, the remaining part of the total hypoxic response and the total glucose response appears to localize to the proximal region of the type II hexokinase promoter, as plasmid F lacking the TATA region shows almost negligible responses to hypoxia and glucose in reporter gene analysis.

The Overlapping E-box/HIF-1 Sequences Located in the Distal Region of the Hepatoma Type II Hexokinase Promoter Are Protected from DNase I by Nuclear Extracts—Nuclear extracts prepared from AS-30D hepatoma cells freshly isolated from the host animal were used in DNase I footprint analysis of the distal region of the type II hexokinase promoter containing the two overlapping E-box/HIF-1 sequences. Significantly, as shown in Fig. 4A, these two sequences were found to be protected, indicating that nuclear proteins (potential transcription factors) are present in this hepatoma cell line that bind at these sites. In addition, results of the DNase I footprint analysis revealed that four other sequences near the two overlapping E-box/HIF-1 sequences also bind nuclear factors. Of these, one is a potential Sp1 element (5’-GTGCAC-3’) that overlaps one of the two overlapping E-box/HIF-1 sequences, whereas two others are identified as potential C/EBP and GATA-1 response elements (Fig. 4B). The fourth protected sequence has no specific identification at present.

**Fig. 2.** A, left panel, SDS-PAGE profile in the 60- to >200-kDa range of the nuclear extract from AS-30D hepatoma cells. Lanes 1 and 2, 4 and 20 μg of nuclear extract. Right panel, Western analysis of AS-30D nuclear extract, also in the 60- to >200-kDa Range. After transfer of the SDS-PAGE gels shown in the right panel to PVDF membranes, they were probed with a HIF-1α monoclonal antibody to the α subunit as described under “Methods.” B, upper panel, SDS-PAGE profile in the 80–200-kDa range for nuclear extracts from AS-30D hepatoma cells exposed to normoxic conditions + pyruvate (1 mM) (lane 1), normoxic conditions + glucose (5 mM) (lane 2), hypoxic conditions + pyruvate (1 mM) (lane 3), or hypoxic conditions + glucose (5 mM) (lane 4). In each well 8 μg of nuclear extract was loaded. Lower panel, Western analysis of AS-30D hepatoma cell nuclear extracts, also in the 80–200-kDa range, following exposure of the cells to the above normoxic or hypoxic conditions. After transfer of the SDS-PAGE gels shown in the upper panel to PVDF membranes, they were probed with a HIF-1α monoclonal antibody to the α subunit.
These data are consistent with the results presented earlier in this report and add additional support for the view that the E-box/HIF-1 overlapping sequences located in the distal region of the hepatoma type II hexokinase promoter play a role in this promoter's response to hypoxia and glucose.

**DISCUSSION**

Results of experiments reported here provide new insights into the mode of regulation of the gene encoding hepatoma type II hexokinase, the predominant hexokinase isoform expressed in poorly differentiated, rapidly growing hepatomas exhibiting the high glucose catabolic phenotype (6–10). Specifically, we have shown that the promoter for this enzyme contains in its distal region two overlapping E-box/HIF-1 sequences separated by about 50 base pairs (Fig. 1B) that are known to be associated in other gene promoters (16–18, 28, 29) with responsiveness to glucose (E-box) or hypoxic conditions (HIF-1). In addition, we have shown that activation of the type II hexokinase gene promoter in transfected hepatoma cells, freshly isolated from the host animal, approaches 7-fold in the presence of hypoxic conditions and a physiological concentration (5 mM) of glucose (Fig. 1C), in concert with this latter finding is the recent result reported by others (32) that, in the A549 human lung cell line, the proximal region of the type II hexokinase promoter also contributes significantly to hexokinase expression under hypoxic conditions.

The apparent synergism between glucose and hypoxic conditions reported here in activating hepatoma type II hexokinase...
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Gene expression has, to our knowledge, not been observed previously for a tumor-associated metabolic gene and deserves further investigation, particularly as it relates to biosynthetic enzymes involved in the rapid production of cell building blocks during tumorigenesis. Thus, for cancer cells to survive within a tumor that is proliferating faster than its vascular system, they must be able to thrive in a hypoxic environment and, in so doing, must not only utilize glucose as the major or sole energy source, but as a major carbon source for biosynthesis as well. Therefore, when faced with such a life or death situation, tumor cells subjected to hypoxic conditions may rely not only on glucose + hypoxic response elements within the promoters of their key glycolytic genes, but in the promoters of their biosynthesis-related genes as well.

The results of studies reported here may be relevant also to tumor metastasis. As indicated in earlier reports (14, 15), tumor cells subjected to hypoxic stress may acquire additional mutations in tumor suppressor genes like p53 leading to the development of a clonal population of more aggressive cancer cells with the capacity to metastasize and ultimately kill the host (14, 15). If such events are found to contribute significantly to tumor metastasis, it seems likely that the gene encoding type II hexokinase that can be markedly activated by hypoxic conditions in the presence of glucose will be involved.

**Fig. 4. A.** DNase I footprint analysis of the distal region of the type II hexokinase promoter harboring the overlapping E-box and HIF-1 sequences. Promoter DNA (–3890 to –703) was footprinted with DNase I in the absence (far left lane) and in the presence of hepatoma nuclear extract (remaining three lanes) using, respectively, from left to right, 5-, 10-, and 20-μg samples exactly as described under “Methods.” The orange boxes on the right designate protected regions, with the predicted overlapping E-box and HIF-1 regions indicated. B, detail of the DNase I footprinted region containing several predicted response elements. Orange boxes above the distal type II hexokinase promoter sequence designate the positions in the footprinted gel in A. Box 1, overlapping Sp1/E-box/HIF-1 response elements; box 2, GATA-1 response element; box 3, CACCC factor response element; box 4, unknown response element; box 5, overlapping E-box/HIF-1 response elements; box 6, CEBP response element.