Regulation of glut1 mRNA by Hypoxia-inducible Factor-1

INTERACTION BETWEEN H-ras AND HYPOXIA*

Oncogenic transformation and hypoxia both induce glut1 mRNA. We studied the interaction between the ras oncogene and hypoxia in up-regulating glut1 mRNA levels using Rat1 fibroblasts transformed with H-ras (Rat1ras). Transformation with H-ras led to a substantial increase in glut1 mRNA levels under normoxic conditions and additively increased glut1 mRNA levels in concert with hypoxia. Using a luciferase reporter construct containing 6 kilobase pairs of the glut1 promoter, we showed that this effect was mediated at the transcriptional level. Promoter activity was much higher in Rat1-ras cells than in Rat1 cells and could be down-regulated by cotransfection with a dominant negative Ras construct (RasN17). A 480-base pair (bp) covalt/hypoxia-responsive fragment of the promoter containing a HIF-1 binding site showed significantly higher activity in Rat1-ras cells than in Rat1 cells, suggesting that Ras might mediate its effect through HIF-1 even under normoxic conditions. Consistent with this, Rat1-ras cells displayed higher levels of HIF1-α protein under normoxic conditions. In addition, a promoter construct containing a 4-bp mutation in the HIF1 binding site showed lower activity in Rat1-ras cells than a construct with an intact HIF1 binding site. The activity of the latter construct but not the former could be down-regulated by RasN17, supporting the importance of the HIF1 binding site in regulation by Ras. The phosphatidylinositol 3-kinase inhibitor LY29004 down-regulated glut1 promoter activity and mRNA levels under normoxia and also decreased HIF1α protein levels in these cells. Collectively these results indicate that H-Ras up-regulates the glut1 promoter, at least in part, by increasing HIF-1α protein levels leading to transactivation of promoter through the HIF-1 binding site.

Oncogenic transformation of mammalian cells is associated with many alterations in metabolism (see Ref. 1 for review). An increased rate of glucose transport is among the most characteristic biochemical markers of the transformed phenotype. The Glut1 glucose transporter is one of the proteins responsible (reviewed in Ref. 2). A number of oncogenes, including fps, src, and ras have been shown to increase glucose transport and to up-regulate glut1 mRNA and protein levels (3–5). Glut1 gene expression and glucose transport are also stimulated in a variety of cells under hypoxic conditions, a response that is mediated by the transcription factor HIF-1. HIF-1 binds to a cis-acting binding sites located within the 5'-flanking region of the glut1 gene (8, 9).

Because hypoxia and oncogenic mutations are both commonly present in tumors, we set out to examine the interaction between the two in up-regulating glut1 mRNA levels. Mutations in Ras are seen in a third of human cancers (6); therefore, as our model system we used Rat1 fibroblasts transformed with H-ras. Transformation with H-ras led to a substantial increase in glut1 mRNA levels under normoxic conditions and additively increased glut1 mRNA levels in concert with hypoxia. Our results indicate that a HIF-1 binding site located in the glut1 promoter is important in its regulation by Ras. We further show that oncogenic H-Ras leads to increased promoter activity by up-regulating the steady-state level of HIF-1α protein and that this involves the PI3K pathway, known to be downstream of Ras (7).

EXPERIMENTAL PROCEDURES

Cell Culture and Establishment of H-ras-transformed Cell Lines and Reagents—Rat1 cells were maintained in culture in DMEM supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals) and cultured in a humidified 95% air/5% CO2 incubator at 37 °C. To establish stable cell lines, Rat1 cells were transfected with an H-ras plasmid using Fugene (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Briefly, Rat1 cells were plated into a 100-mm dish 24 h before transfection. Cells were transfected using 6 μl of Fugene and 2 μg of the plasmid pAL8-H-ras, which contains the H-ras genomic DNA and a neomycin-resistance gene. Transfected cells were selected in DMEM supplemented with 400 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) for 1 week. Colonies were isolated using cloning cylinders, trypsinized, then expanded as cell lines. The expression of the H-ras gene was confirmed by Western blotting with an anti-H-Ras monosclonal antibody. Twelve G418-resistant colonies were expanded, all of which expressed high levels of H-Ras protein compared with the Rat1 parental cells. We used clone 12, which expressed approximately 10-fold the level of H-Ras as the parental Rat1 cells (data not shown) for most of our experiments. Rat1-ras cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 400 μg/ml G418. PD098059 and LY294002 (from Alexis Biochemicals, San Diego, CA) were dissolved in Me2SO at concentrations of 100 mM and 40 mM, respectively.

Northern Blot Analysis—Total RNA was isolated with TRIzol (Life Technologies Inc.) following the manufacturer’s instructions. 5–10 μg of total RNA was run on a 0.9% agarose gel containing formaldehyde.

Received for publication, November 7, 2000

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Printed in U.S.A.
transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA), and UV cross-linked prior to hybridization. Labeling of radioactive DNA probes was performed using [32P]dCTP and a Prime-It kit (Stratagene). Hybridization was carried out at 65 °C, after which the membranes were washed to a stringency of 0.1 × SSC, 0.1% SDS at 65 °C. Autoradiography was carried out at −80 °C with intensifying screens. A 400-bp mouse glut1 cDNA fragment (from M. Birnbaum, University of Pennsylvania) was used to make radioactive probes.

**Western Blot Analysis**—For protein isolation, cells were washed once with cold phosphate-buffered saline containing 1 m EDTA, then solubilized by adding lysis buffer (1% Triton X-100, 200 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM orthovanadate, 2 mM phenylmethylsulfonyl fluoride) directly on the cells. The cell lysate was transferred into 1.5-mL microfuge tubes and centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were transferred to a fresh tube and frozen on dry ice. Protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL). For Western blotting, an equal amount of total protein was separated by SDS-polyacrylamide gel electrophoresis on a 6% polyacrylamide gel. After completion of gel electrophoresis, protein was transferred onto a Transblot nitrocellulose membrane (Bio-Rad, Hercules, CA) using a blotting apparatus. For detection of H-Ras protein, we used an anti-human H-Ras monoclonal antibody (Qualitex Biotech, Camden, NJ) at a dilution of 1:1000 followed by a secondary goat anti-mouse antibody (Bio-Rad). Antibody binding was detected by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**Hypoxic Conditions**—For hypoxia experiments, 5–7 × 10^5 cells were plated on day 1 into 60-mm Pernox dishes (Nunc) and maintained in a 5% CO₂ incubator. Pernoxo plastic was used because of its high permeability to oxygen, permitting the efficient evacuation of oxygen from the dishes before hypoxia induction. On day 2, immediately before the induction of hypoxia, standard media was replaced with media containing 50 mM HEPES, 0.15% (w/v) glucose, and 10 mM NaOH to maintain pH. Dishes were then placed into airtight aluminum chambers. The oxygen concentration was decreased by sequentially replacing a given percentage of the total gas within a chamber with 95% nitrogen/5% CO₂ using a precision vacuum gauge. The oxygen concentration was reduced from 21% to 5% with one gas exchange, from 5% to 1% with a second gas exchange, then with an additional gas exchange to 0.2%. The aluminum chambers were then placed on an orbital shaker in a warm room maintained at 37 °C.

**Plasmid Constructs, Transient Transfections, and Luciferase Assay**—A series of constructs containing various fragments from the 5'-flanking region of the rat glut1 gene were used. Constructs A, C, E, and K are described elsewhere (8). As shown in Fig. 2 (see below), constructs A, C, and E contain, respectively, 6, 3.5, and 3 kb of the 5'-flanking region of the rat glut1 gene. Construct K contains a 480-base pair sequence from the 5'-region of the rat glut1 promoter located at −3.5 to −3.0 kb relative to the transcription start site (GenBank accession number U82755). This 480-bp enhancer contains a potential HIF-1 binding site at nucleotides +398 to +401.

Construct N was made by PCR amplification in the following manner. The primer pair (1A, 5'-TAATAGTTCCCAACAGAGCCTTTC-3'; 2B, 5'-TAATAGTGTAAGGATCTCCAGGAACTTG-3') was used for amplifying using construct K as a template. Construct N contains 184 bp of the glut1 promoter located at approximately −3.2 to −3.0 kb relative to the transcription start site (from nucleotides +297 to +480; GenBank accession number U82755). These primers were chosen to eliminate the SRE (at nucleotides +287 to +296), which has been shown to have a positive role in stimulation of the glut1 promoter (8), while retaining the potential HIF-1 binding site (nucleotides +398 to +401). Primer 1A has incorporated within it a KpnI restriction site and primer 2B an NheI restriction site, so the PCR product could be restricted with these two enzymes and subcloned into the KpnI/NheI sites of the reporter plasmid pGL3-Promoter. pGL3-Promoter contains its own SV-40 promoter, which was required because the fragment that was PCR-amplified lacks the glut1 TATA box and transcription start site. Construct M was made using a two-step overlapping PCR strategy. In step one, two separate pairs of primers were used in PCR amplification, primer 1A (listed above) and primer 1B, 5'-TGGCGTGCAGGCA- GACATCGTCG-3' in one reaction, and primer 2A, 5'-CACAGGAT GTCTGCGTACGACCA-3' and primer 2B (listed above) in the second.

**RESULTS**

**H-ras and Hypoxia Additively Increase glut1 mRNA Levels**—To examine how oncogenic transformation by human H-ras affects expression of the glut1 gene, we stably transfected Rat1 cells with an H-ras expression vector. We compared the expression of glut1 mRNA in Rat1 cells relative to Rat1-ras cells. Rat1 cells expressed very low levels of glut1 mRNA but exhibited a 2.8-fold increase in glut1 mRNA levels after 6 h of hypoxia (Fig. 1; lanes 1 versus 3). In contrast, Rat1-ras cells exhibited increased levels of glut1 mRNA under normoxic conditions (2.5-fold over Rat1 cells; lanes 2 versus 1) and showed an additional 2-fold induction after 6 h of hypoxia (lanes 4 versus 2). Thus, H-ras overexpression both increases the basal level of glut1 mRNA and interacts with hypoxia to additively increase the level of this message. Similar results were obtained using a second independent H-ras transformed subclone (data not shown).

**Ras and Hypoxia Increase glut1 Promoter Activity**—To investigate whether the increased expression of glut1 mRNA by H-ras and hypoxia was due to increased promoter activity, we transfected a rat glut1 promoter-luciferase construct (construct A: “A” in Fig. 2) into Rat1-ras and Rat1 cells. 24 h after transfection, half the dishes were subjected to hypoxia (0.2% oxygen) for 6 h and half were maintained in 21% oxygen. Subsequently cells from all the dishes were harvested and assayed for luciferase activity. In Rat1 cells there was a 2.2-fold induction of the
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glut1 promoter by hypoxia (Fig. 3A). Rat1-ras cells showed a 9-fold higher luciferase activity than Rat1 cells under normoxic conditions and exhibited a further 3.3-fold increase when exposed to hypoxia (Fig. 3A). To demonstrate that the glut1 promoter activity in Rat1-ras cells was specifically due to the effect of H-Ras, we cotransfected a construct expressing dominant negative RasN17 into these cells, which resulted in a decrease in glut1 promoter activity (Fig. 3B). Thus, the activity of the glut1 promoter is increased in Rat1-ras cells compared with Rat1 cells, and it can be further increased by hypoxia.

Analysis of the 5' Flanking Region of the glut1 Promoter—To better characterize the elements in the 5' flanking region of the rat glut1 gene responsive to H-Ras, a series of deletion mutants of the promoter was used in transient transfection experiments in both Rat1 and Rat1-ras cells under normoxia and hypoxia (Fig. 4). glut1 promoter activity was weak in the Rat1 parental cells with all of the constructs tested. There was approximately a 2-fold induction of promoter activity by hypoxia with constructs A, C, and K, but only a 1.3-fold induction with construct E, which lacks a potential HIF-1 binding site. Similar to results shown in Fig. 3, in Rat1-ras cells, the basal level of glut1 promoter activity with construct A was 5-fold greater than in Rat1 cells, and hypoxia led to a 3.5-fold induction of activity compared with normoxia in Rat1-ras cells. Construct K gave a higher level of expression in Rat1-ras cells under normoxia or hypoxia that either construct A or C, possibly due to the fact

FIG. 2. Glut1 promoter constructs. A, a series of promoter deletion constructs were derived from the ~6-kbp rat GLUT1 promoter. Constructs A, C, E, and K (letters A, C, E, and K) have been previously described (19). Construct A contains the entire 6-kb 5'-flanking region of the rat glut1 gene in which there are two hypoxia-inducible elements, a 660-bp BglII/PstI azide-responsive segment, located between approximately −6.0 and −5.3 kbp relative to the transcription start site and a 480-bp PstI/SacI cobalt-responsive element, located between approximately −3.5 and −3.0 kbp. Construct C contains a 5'-deletion but still retains the 480-bp cobalt-responsive element, which contains both an SRE and a HIF-1 binding site. Construct E lacks the 480-bp cobalt-responsive element. Constructs A, C, and E were made using the luciferase reporter vector pGL2-Basic. The 480-bp cobalt-responsive element was subcloned into a luciferase reporter construct containing the c-fos promoter to make construct K. Construct N was made as described under “Experimental Procedures” and contains a 184-bp sequence from the glut1 promoter lying approximately −2.4 to −2.2 relative to the transcription start site (spanning nucleotides +297 to +480; GenBank accession number U82755). Within this 184-bp sequence is located the HIF-1 binding site (from nucleotide +398 to +402; GenBank accession number U82755); however, the SRE has been deleted. Construct M is identical to construct N except for a 4-bp mutation in HIF-1 binding site. Both construct M and N were subcloned into the reporter pGL3-Promoter, which contains its own SV40 promoter. To further characterize the elements in the 5'-flanking region of the glut1 promoter, a series of deletion mutants was cotransfected into Rat1 and Rat1-ras cells, and it can be further increased by hypoxia.

FIG. 3. Effect of H-ras on glut1 promoter activity. A, cells were plated onto a 60-mm dish the day before transfection. The following day, cells were transfected with 2 μg of construct A (Fig. 2) and 1 μg of pSV-β-galactosidase (Promega). 24 h later, culture media was replaced with buffered media and the cells were subjected to 6 h of hypoxia (0.2% oxygen) or normoxia before they were collected and analyzed for luciferase and β-galactosidase. B, Rat1-ras cells were transfected with 2 μg of construct A along with 1 μg of pSV-β-galactosidase (Promega). Along with these plasmids was cotransfected either 0.5 μg of pcDNA3 or 0.2 μg of pcDNA3/RasN17. For both A and B, normalized luciferase levels (ratio of luciferase to β-galactosidase readings) are plotted on the y axis. Values represent the mean of three independent transfections. The error bars represent one S.D. from the mean.
H-Ras might increase the level of the HIF-1 binding site in the glut1 promoter/luciferase constructs. This effect was important for its regulation by H-Ras, we speculated that H-Ras might increase the level of the HIF-1 protein. We found that Rat1-ras cells contained higher levels of the protein under both normoxic and hypoxic conditions than did Rat1 cells (Fig. 6). This figure also shows that cobalt chloride, which is a hypoxia mimic, leads to higher steady-state levels of HIF-1α in Rat1-ras cells than Rat1 cells.

FIG. 4. Activity of glut1 promoters in Rat1 and Rat1-ras cells in normoxia and hypoxia. glut1 promoter constructs (Fig. 2) were tested for hypoxia responsiveness in Rat1 and Rat1-ras cells. Cells were transfected with 2 μg of reporter plasmid (either construct A, C, E, or K) and 1 μg of pSV-β-galactosidase, allowed to recover for 24 h, exposed to air or hypoxia for 6 h, then assayed for luciferase and β-galactosidase activity. Normalized luciferase levels (ratio of luciferase to β-galactosidase readings) are plotted on the y axis. Values represent the mean of three independent transfections. The error bars represent one S.D. from the mean.

The HIF-1 Binding Site in the glut1 Promoter Is Important for Up-regulation by Ras under Normoxic Conditions—The glut1 promoter is known to contain a HIF-1 binding site, which is required for its transactivation by hypoxia through the transcription factor HIF-1 (9). To test whether this site is important for transactivation of the promoter by H-Ras under normoxic conditions we made a pair of reporter constructs, construct N, which contains 184 bp of the promoter, including the HIF-1 binding site, and construct M, which is identical except for a 4-bp mutation, which abolishes the HIF-1 binding site (Fig. 2). As expected, construct N (Fig. 5A) but not construct M (Fig. 5B) was inducible by hypoxia. We performed transient transfections of both constructs into Rat1-ras cells with and without cotransfection of a dominant negative RasN17 expression vector. Fig. 5C shows that the basal promoter activity using construct M, which has a mutated HIF-1 binding site, was 37% less than that of construct N, which has an intact HIF-1 binding site. In two additional independent experiments we found the promoter activity of construct M to be 27 and 33% less than the activity of construct N (data not shown). Fig. 5C also shows that promoter activity is inhibited by ~40% in cells cotransfected with construct N and RasN17, whereas RasN17 had no effect on luciferase expression in cells transfected with construct M. These results support the premise that H-Ras exerts a positive effect on the glut1 promoter transcription through the HIF-1 binding site.

Ras Increases HIF-1α Protein Levels and HIF-1 Binding—Having found that the HIF-1 binding site in the glut1 promoter was important for its regulation by H-Ras, we speculated that H-Ras might increase the level of the HIF-1α protein. We found that Rat1-ras cells contained higher levels of the protein under both normoxic and hypoxic conditions than did Rat1 cells (Fig. 6). This figure also shows that cobalt chloride, which is a hypoxia mimic, leads to higher steady-state levels of HIF-1α in Rat1-ras cells than Rat1 cells.

Both the MAP Kinase Inhibitor PD098059 and the PI3K Inhibitor LY294002 Down-regulate glut1 mRNA Expression and Promoter Activity and HIF-1α Levels—Both the PI3K and MAP kinase pathways have been shown to be activated by Ras (7). Therefore, to define the signal transduction pathways through which expression of Glut1 is regulated by Ras, we transiently transfected cells with glut1 promoter/luciferase constructs (either construct A or K), then treated them with either a PI3K inhibitor (LY294002) or an inhibitor of MAP kinase (PD098059) under normoxic or hypoxic conditions before harvesting cells for luciferase assays. PD098059 and LY294002 both down-regulated glut1 promoter activity using construct A in Rat-ras cells under normoxic conditions, and both inhibited the induction of glut1 mRNA by hypoxia (Fig. 7).
FIG. 6. HIF-1α levels in Rat1 and Rat1-ras cells. Rat1 and Rat1-ras cells were subjected to normoxia or hypoxia (0.2% oxygen) for 6 h, then cells were harvested in protein lysis buffer. Samples were run on a 6% polyacrylamide gel, then Western blotting was performed. Blot was probed with anti-HIF-1α primary antibody and goat anti-mouse secondary antibody.

FIG. 7. Effect of PD098059 and LY294002 on glut1 promoter activity. Cells were transfected with 2 µg of reporter gene. 24 h later cells were treated with PD098059 (50 µM) or LY294002 (40 µM) then subjected to normoxia or hypoxia (0.2% oxygen) for 6 h. Cells were then collected and assayed for luciferase activity. Normalized luciferase levels (ratio of luciferase to β-galactosidase readings) are plotted on the y axis. Values represent the mean of three independent transfections. The error bars represent one S.D. from the mean.

Similar results were obtained using construct K (Fig. 7). Therefore, both PI3K and MAP kinase inhibition have a negative effect on the glut1 promoter. To determine whether they have an effect on glut1 mRNA levels, Rat1-ras cells were treated with either inhibitor under normoxic or hypoxic conditions before harvesting cells for RNA. Under normoxic conditions, treatment with either inhibitor led to a decrease in glut1 mRNA levels (Fig. 8; compare lanes 3 and 4 with lane 2), and treatment with both drugs led to a greater decrease in the level (Fig. 8; compare lane 5 with 2). We also treated Rat1-ras cells with bisindolylmaleimide I, a protein kinase C inhibitor, and found that this had no effect on VEGF mRNA levels (data not shown). We then treated Rat1-ras cells with the inhibitors prior to exposure to hypoxia. Neither drug alone had a detectable effect on glut1 message level (Fig. 8; compare lanes 8 or 9 with lane 7); however, using both drugs together reduced glut1 mRNA levels by ~30% (Fig. 8; compare lane 10 with lane 7).

To determine whether the effect of PD098059 and LY294002 in decreasing glut1 mRNA levels in normoxia might be mediated through HIF-1α, we examined HIF-1α levels in Rat1-ras cells after drug treatment. Fig. 9 shows that treatment with LY294002 led to a decrease in the level of HIF-1α, whereas treatment with PD098059 had no such effect. Two additional independent experiments confirmed this result (data not shown).

DISCUSSION

For decades it has been known that tumors display numerous metabolic changes compared with normal cells. Tumors frequently display increased glycolytic metabolism even under aerobic conditions, an effect named after Warburg who originally described it (10). To keep up with this increased glycolysis, there must be an increased uptake of the substrate, glucose. Human tumors have been shown to have increased glucose uptake in vivo compared with normal tissues (see Ref. 11 and references within). One of the proteins responsible for the uptake of glucose into cells is Glut1, which was the first of a family of glucose transporters to be cloned (12). Glut1 is expressed in most normal tissues and is responsible for basal glucose transport. Numerous factors have been shown to regulate glut1 mRNA expression, including phorbol esters, oncogenes, hypoxia, growth factors, and mitogens (reviewed in Refs. 2 and 13). Over a decade ago it was observed that transformation of rodent fibroblasts by Fujiyama sarcoma virus (4) or by the ras or src oncogenes (3) resulted in a marked increase in glucose uptake, which was accompanied by an increase in Glut1 protein and mRNA expression. In the case of Fujiyama sarcoma virus, it was shown that the increase in glut1 mRNA occurred at the level of transcription (4). Glut1 levels have been found to be increased in a variety of human cancers (14, 15). One study found that Glut1 expression increased with increasing grade of malignancy in human colon cancers and was as-
associated with a higher proportion of lymph node metastases (14).

Cells subjected to hypoxia must undergo metabolic adaptations to survive. Hypoxia induces the expression of many genes to allow for this to occur (reviewed in Refs. 16 and 17). Part of this adaptation to hypoxia involves up-regulation of genes that encode the enzymes required for anaerobic glycolysis, thus allowing cells to switch to this form of metabolism from oxidative phosphorylation. Under hypoxic conditions there is also a parallel increase in glucose uptake, which is facilitated by up-regulation of Glut1 expression. Hypoxia is a potent stimulus for glut1 mRNA induction in a variety of tissue types, including endothelial cells (18), hepatic cells (19), various tumor cell lines (9), and alveolar epithelial cells (20). The mechanism of glut1 message induction by hypoxia is complicated and is usually controlled by low oxygen concentrations per se and by inhibition of oxidative phosphorylation (19). The former can be mimicked by covalent chloride and the latter by sodium azide. The covalent-responsive element in the rat glut1 promoter has been mapped and is homologous to the mouse Enhancer-1 sequence (9, 19). Transactivation through this element in the promoter is mediated by HIF-1 (9) and a heterodimeric transcription factor that binds to a specific DNA consensus sequence, 5′-RCGTTG-3′, found in the promoters of many hypoxia-inducible genes (for review see Ref. 16). The HIF-1 transcription factor consists of two subunits, HIF-1α and HIF-1β, both of which are basic helix-loop-helix proteins. HIF-1α is a component of several transcription factors, and its protein level is not significantly induced by hypoxia. In contrast, HIF-1α protein, which is unique to the HIF-1 complex, is rapidly degraded in oxygenated cells by the ubiquitin-proteasome pathway (21). HIF-1α protein is specifically induced by hypoxia in a graded response dependent on the oxygen concentration (22).

Although the mechanism by which hypoxia increases glut1 mRNA expression through HIF-1 is relatively well understood, it is less clear how oncogenes such as Ras increase glut1 mRNA levels. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate, which activates protein kinase C, is known to increase glut1 mRNA levels (23); however, Glut1 induction by growth factors and oncogenes such as K-ras occurs via a protein kinase C-independent pathway (23, 24). We were interested in understanding how a specific oncogene, mutant H-ras, led to increased glut1 mRNA levels and in studying its interaction with hypoxia in regulating message expression. For our model system we used Rat1 cells transformed with this oncogene. We found that H-ras transformation increased the basal level of glut1 mRNA under normoxia compared with Rat1 cells, consistent with what others have found using other oncogenes (3, 4). We also found that H-ras interacts additively with hypoxia to increase mRNA expression (Fig. 1). Furthermore, the effect of Ras on glut1 mRNA levels is mediated at the level of the promoter, since the promoter activity is greater in Rat1-ras cells than in Rat1 cells (Fig. 3A).

In analyzing the 5′-flanking region of the glut1 gene using deletion constructs in transient transfection experiments, we found that construct K, which contains a HIF-1 binding site, was sufficient to mediate up-regulation of promoter activity by H-ras. However, construct K contains a 480-base pair sequence from the 5′-region of the rat glut1 promoter located between −3.5 and −3.0 kb relative to the transcription start site. In addition to a HIF-1 binding site (nucleotides +398 to +401; GenBank® accession number U82755), this 480-bp stretch contains sites for several other transcription factors, including AP-1 binding sites (at nucleotides +248 to +254 and +320 to +327) and an SRE (at nucleotides +287 to +296), which have been previously implicated in the regulation of this promoter (8). Therefore, to rigorously test the importance of the HIF-1 binding site, we created reporter constructs M and N, neither of which contain the SRE. These constructs contain 184 bp of the glut1 promoter located from approximately −3.2 to −3.0 kb relative to the transcription start site (from nucleotide +297 to +480; GenBank® accession number U82755). Constructs M and N are identical except that the former contains a 4-base pair mutation of the HIF-1 binding site. Therefore, as expected, construct N was inducible by hypoxia, but not construct M. Both constructs showed activity in Rat1-ras cells under normoxic conditions, but a key difference is that the activity of construct N, but not construct M, could be down-regulated by mutant RasN17. Therefore, this strongly suggests the HIF-1 binding site is important in regulation by H-Ras. Construct M also exhibited lower basal luciferase activity in Rat1-ras cells than did construct N, between 25 and 40% less. We speculate that the reason that the difference is not greater is because both of these constructs (M and N) are driven by an SV40 minimal promoter rather than the TATA box intrinsic to the glut1 promoter; therefore, there is a high basal level of activity.

The 480-bp sequence for the rat glut1 5′-region shows significant homology to the 5′-Enhancer-1 region of the mouse glut1 gene that was cloned by Murakami et al. (8). This group found that the mouse glut1 Enhancer-1 was inducible by H-Ras. They showed that the SRE and two AP-1 sites within this enhancer were important in promoter activity, although they did not specifically examine the role that these sites played in regulation by Ras. However, they found that these three elements were insufficient for full basal activity of Enhancer-1. Subsequently, the HIF-1 binding site within the mouse glut1 Enhancer-1 was identified (9). Our data indicate that this HIF-1 binding site is important in the regulation of the rat glut1 promoter by H-Ras. Of note, activation of the mouse glut1 promoter by oxidative stress in L6 myotubes was shown to involve both the SRE and the AP-1 binding sites in Enhancer-1 but was independent of the Ras/MAP kinase pathway (25).

How does H-Ras regulate activity of the HIF-1 binding site in the glut1 promoter? One mechanism is by increasing the level of HIF-1α protein. Rat1-ras cells contain higher levels of the protein than do Rat1 cells under both hypoxic and normoxic conditions. Although HIF-1α was originally described as a transcription factor, which was undetectable under normoxic conditions and inducible only under hypoxia (26), there is accumulating evidence that it is also important in gene regulation under normoxic conditions. Src-transformed cells have been found to contain higher levels of HIF-1α under normoxic conditions than nontransformed parental cells (27). HIF-1α has been found to be induced in tumor cell lines by a variety of hormones and growth factors, including epidermal growth factor, insulin-like growth factor-1, insulin, and angiotensin II (28–31).

Both inhibitors of the MAP kinase and the PI3K pathways led to decreased glut1 promoter activity and glut1 mRNA levels under normoxia. In contrast, neither drug by itself appeared to block the induction of glut1 mRNA by hypoxia, although the two together did have an inhibitory effect. This apparent discrepancy between the effects of these inhibitors on glut1 message under normoxia and hypoxia might be explained by the fact that the induction of glut1 mRNA by hypoxia involves mRNA stabilization in addition to transcription of the promoter (19). Therefore, in transfection experiments using promoter constructs, we are specifically examining transcriptional regulation, whereas in the Northern blots, the results are complicated by the fact that RNA stabilization, which may be independent of PI3K and MAP kinase, also plays a role.

Even though we found that both MAP kinase and PI3K
inhibitors decreased *glut1* mRNA promoter activity, only the latter led to a detectable decrease in HIF-1α protein levels. A possible role for the PI3K pathway in regulating HIF-1α protein levels has been suggested in the context of PTEN expression in glioblastoma cells (32) and epidermal growth factor stimulation in prostate carcinoma cells (29). In regard to MAP kinase, both p42 and p44 MAP kinase have been shown to phosphorylate HIF-1α *in vitro* and to stimulate HIF-1α transcription activity without affecting the level of the protein (33). Therefore, it is conceivable that the drug PD098059 might decrease *glut1* mRNA levels and promoter activity through an effect on HIF-1α function without affecting the level of the protein itself.

Our results suggest a link between H-ras transformation, HIF-1α up-regulation, and *glut1* mRNA transactivation. In the future we plan to pursue this path of investigation to understand how H-Ras leads to HIF-1α up-regulation. Although we have examined the expression of a specific gene, *glut1*, as a downstream target of H-Ras, it is likely that the up-regulation of other hypoxia-inducible genes such as VEGF occurs by a similar mechanism. A number of groups have shown that mutant Ras leads to increased VEGF levels in diverse cell types (34–36). In NIH3T3 cells, activated H-Ras has been postulated to increase VEGF reporter expression only when an intact HIF-1 binding site is present (37). Thus, mutations in H-ras can not only lead to transformation but may also help cells survive in a hypoxic environment by increasing the expression of specific genes through up-regulation of HIF-1α.

Acknowledgments—We are grateful to Don Solomon for technical assistance and Dr. Xiaoling Su for her help with data analysis. We thank Eric Bernhard, Frank Lee, and Ruth Muschel for reading the manuscript and offering helpful comments. We are grateful to Amita Sehgal for use of the TopCount Microplate scintillation and luminescence counter.

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