Differential Gene Up-Regulation by Hypoxia-Inducible Factor-1α and Hypoxia-Inducible Factor-2α in HEK293T Cells

Victoria Wang,David A. Davis,Muzammel Haque,L. Eric Huang and Robert Yarchoan

Abstract

Cells exposed to hypoxia respond by increasing the level of hypoxia-inducible factor-1 (HIF-1). This factor then activates a number of genes by binding to hypoxia response elements in their promoter regions. A second hypoxia-responsive factor, HIF-2, can activate many of the same genes as HIF-1. Overexpression of HIFs accompanies the pathogenesis of many tumors. It is unclear, however, as to the respective role of these factors in responsiveness to hypoxia and other stresses. To address this issue, we used microarray technology to study the genes activated in HEK293T cells by hypoxia or transfection with the α chain of HIF-1 (or mutant HIF-1 resistant to degradation) or HIF-2. Fifty-six genes were found to be up-regulated at least 3-fold by either hypoxia or transfection. Of these, 21 were elevated both by transfection with HIF-1α and with HIF-2α, and 14 were preferentially activated by HIF-1α including several involved in glycolysis. Ten genes were preferentially activated by HIF-2α, including two (CACNA1A and PTPRZ1) implicated in neurologic diseases. Interestingly, most HIF-2α-responsive genes were not substantially activated by hypoxia. An additional 10 genes were up-regulated by hypoxia but minimally activated by HIF-1α or HIF-2α transfection. Ten of the genes were studied by quantitative real-time PCR and/or by Northern blot and the results paralleled those found with microarray technology. Although confirmation in other systems will be necessary, these results indicate that whereas some genes are robustly activated by both HIF-1 and HIF-2, others can be preferentially activated by one or the other factor. (Cancer Res 2005; 65(8): 3299-306)

Introduction

Cells must be able to rapidly respond to sudden changes in oxygen levels to maintain energy-dependent activities and survive in response to the stress of reduced oxygen tension (hypoxia). Temporary shortages of oxygen lead to the depletion of cellular ATP, whereas sustained deprivation of oxygen can cause irreversible damage and cell death (1). A number of systemic and cellular responses have developed to enable organisms to respond to hypoxia, including glycolysis, angiogenesis, vasodilation, and erythropoiesis (2). Tumors commandeer these mechanisms to adapt to the hypoxia that can develop when their rapid growth outstrips the blood supply.

Many of the adaptations to hypoxia are mediated by the activation of specific genes through the action of hypoxia-inducible factor (HIF). The first HIF described (called HIF-1) is a heterodimer made up of HIF-1α and HIF-1β (3–5). HIF-α, a basic helix-loop-helix transcription factor, is constitutively expressed (6), but under normoxic conditions is hydroxylated at specific proline residues resulting in ubiquitination through the interaction with von Hippel-Lindau factor suppressor protein (pVHL) and proteasomal degradation (7, 8). Under hypoxic conditions, proline hydroxylation is inhibited, preventing association with pVHL. HIF-1α accumulates and associates with HIF-1β to form a heterodimer, which accumulates in the nucleus and activates a specific set of genes by binding to hypoxia response elements in the promoter region of these genes (9).

A structurally related protein, HIF-2α, has been described and partially characterized (10). HIF-2α (also known by the names EPAS1, HRF, HLF, and MOP2) shares ~48% overall amino acid identity with HIF-1α and 83% identity in the basic helix-loop-helix domains (10, 11). Like HIF-1α, HIF-2α accumulates in the presence of hypoxia, forms a heterodimer with HIF-1β (called HIF-2), and binds to hypoxic response elements. HIF-2α has been shown to regulate a number of the same hypoxia-inducible genes as HIF-1 (11–16). Given these similarities, the question has arisen as to whether HIF-2α is a redundant protein that activates the same set of genes as HIF-1α or if it has a more specific role either in activating a different subset of genes or in regulating these genes in certain cell types.

Some of the evidence to date has suggested that whereas HIF-1 has broad activity, the response to HIF-2 is restricted to specific cell types (15). Expression of HIF-2 was initially thought to be limited to vascular endothelial cells during embryonic development (10). However, it has since been reported that HIF-2 is expressed in a number of cell types including kidney fibroblasts, hepatocytes, intestinal epithelial cells, pancreatic interstitial cells, heart myocytes, and lung type II pneumocytes (17, 18). Another study using short interfering RNA technology to inhibit HIF-1α or HIF-2α suggested that the relative importance of the genes could vary with the cell type (19); the response to hypoxia was largely mediated by HIF-1α in endothelial and breast cancer cells but by HIF-2α in renal carcinoma cells. More recent studies have suggested that HIF-2 overexpression is important in the development of renal carcinoma in patients with VHL and that in such a setting, HIF-2 may act as a renal cancer oncogene (20, 21). Thus, there is accumulating evidence that the relative importance of HIF-1 and HIF-2 in the response to hypoxia varies among different cell types.

A related question is whether certain genes are preferentially activated by HIF-1 or HIF-2 in those cells in which the factors are active. Our interest in this question arose from our earlier work with Kapoúi’s sarcoma-associated herpesvirus, in which it seemed that two viral genes studied (Rta and ORF-34) differed in their responsiveness to HIF-1α and HIF-2α (22, 23). A number of reports have provided evidence that, in general, HIF-1 and HIF-2 do not differ with respect to their target genes. A notable exception to this was the recent finding by Hu et al. (12) that the up-regulation of
several glycolytic genes by hypoxia in multiple cell types was mediated by HIF-1, but not by HIF-2. However, these authors did not identify any genes in which the response to hypoxia was specifically mediated by HIF-2. In the present study, we have attempted to further address the question as to whether certain genes are preferentially up-regulated by HIF-1 or by HIF-2. Using microarray technology, we have now analyzed the relative up-regulation of various cellular genes in human embryonal kidney cells transiently transfected with either HIF-1 or HIF-2 and compared these results to the gene up-regulation induced in the same cell line under conditions of hypoxia.

Materials and Methods

Cell culture. HEK293T human embryonic kidney cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM supplemented with 10% fetal bovine serum (heat inactivated, Hyclone, Logan, UT) and 1 × antibiotics/glutamine (100 units/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml l-glutamine sulfate, all from Invitrogen Corp., Carlsbad, CA), under either hypoxic (1% O₂) or normoxic (21% O₂) conditions at 37 °C in a tissue culture incubator.

Plasmids and transfections. Plasmids encoding HIF-1α or HIF-2α were gifts from Dr. Steven L. McKnight (University of Texas, Austin, TX; ref. 10). A plasmid encoding a HIF-1α mutation that is resistant to proteosomal degradation (HIF-1α mut) has been previously described (23, 24). All the plasmids were purified with the Qiagen Maxiprep kit (Qiagen, Valencia, CA). The inserts were verified by restriction mapping.

One day before transfection, 5 × 10⁶ HEK293T cells were plated on a 10 cm diameter dish. Confluent cells (60-70%) were then transfected with 20 μg plasmid DNA using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. The transfected HEK293T cells were incubated at 21% O₂ for 48 hours. In previous studies, we found that transfection rates of 60% to 70% are attainable with HEK293T cells using such methodology.

Immunoblotting. Nuclear extracts were prepared from the untransfected HEK293T negative control cells and HEK293T cells transfected with plasmids as well as HEK293T treated in hypoxia for 16 hours using a nuclear extraction kit (Pierce, Rockford, IL). Nuclear extracts (40 μg) were electrophoresed on precast 4% to 12% Tris-Bis NuPAGE gels (Invitrogen), transferred to nitrocellulose membrane, blocked overnight at 4 °C with 5% w/v nonfat dry milk in 1 × TBST (100 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween 20; ref. 23). The blots were then incubated for 1 hour with antibodies to HIF-1α or HIF-2α (Novus Biological, Inc., Littleton, CO). After incubation with a secondary antibody conjugated to alkaline phosphatase for 30 minutes, bands were visualized with the stabilized Western Blue substrate (Promega, Madison, WI).

RNA isolation and Northern blot analysis. Total RNA was extracted from HEK293T cells after 16 hours of normoxic or hypoxic exposures and 48 hours posttransfection with the RNeasy kit (Qiagen) according to the protocol of the manufacturer. Microarrays of total RNA were separated by electrophoresis in 1% denaturing formaldehyde-agarose gel. The RNA was then transferred to nylon membrane (Hybond N+, Amersham Biosciences, Piscataway, NJ) overnight by capillary elution and UV cross-linked. The blots were first prehybridized for 1 to 2 hours at 50 °C in digoxigenin easy hybridization solution to block the nonspecific hybridizations (Roche Applied Science, Indianapolis, IN). Digoxigenin-labeled PCR probes (PCR digoxigenin probe synthesis kit, Roche) were used to hybridize the membrane at 50 °C for 18 hours according to the manufacturer's protocol. The blots were washed, blocked, and incubated with anti-digoxigenin-AP antibody for 30 minutes. After the wash and detection steps (CDP-Star kit, Roche), the membranes were exposed to radiographic films. DNA fragments were synthesized through reverse transcription-PCR (RT-PCR) and subcloned into pCR2.1-TOPO vector to use as templates for Northern blot probes. PCR primers were designed as shown in Table 1.

Oligo microarray and bioinformatic analysis. For oligo microarray experiments, total RNA samples were extracted from HEK293T cells grown in 1% or 21% O₂ overnight and from transfected cells. Cultures were set up and RNA extracted on three separate occasions. Total RNA (20 μg) from each sample was synthesized into double-stranded cDNA with reverse transcriptase (Fairplay labeling kit, Stratagene, La Jolla, CA) using an oligo d(T) primer. The double-stranded cDNA from untreated cells was labeled with Cy5 monofunctional reactive dye and that from hypoxia-treated or transfected cells was labeled with Cy5 monofunctional reactive dye (both from Amersham Biosciences). The probe was hybridized to a long oligo array (Hs-Operon v2.1px.gal) containing 20 K human transcripts [National Cancer Institute (NCI) Microarray Facility, Advanced Technology Center, Gaithersburg, MD] overnight at 42 °C. A description of the oligo libraries is available at http://nciar.cancer.gov/. For each treatment, the arrays were also queried with probes produced via reverse labeling, and the data were consistent with that obtained with the initial standard labeling.

Microarray slides were scanned with a GenePix 4000 microarray scanner (Axon Instruments, Union City, CA). The microarray images were analyzed with GenePix 3.0 software and data analysis was done with the MicroArray Database system hosted by the Center for Information Technology and Center for Cancer Research at the NCI. For each gene, the relative fluorescence intensity of the two signals (hypoxia or transfection versus normoxia) was converted into a digital ratio. Single spots or areas of the array with obvious blennishes or low-quality fluorescence spots were flagged and excluded from subsequent analyses (26). Nonflagged array elements with fluorescence intensity in each channel more than twice of the local background were used. The raw data were uploaded into a custom database (MicroArray database). Fluorescence ratios were normalized for each array by applying a single scaling factor so that the median fluorescence ratio of well-measured spots on each array was 1.0. Spots were additionally excluded

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<td>Reverse: AAAGTGTCGTTGGG AGGTTGTC</td>
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<td></td>
<td>Reverse: CACCTCCCCAGAGTCT ATTTTCC</td>
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<td></td>
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<td></td>
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Quantitative real-time PCR. Total RNA was isolated from HEK293T cells as described above. The RNA was treated with RNase-free DNase I (Ambion, Inc., Austin, TX). First-strand cDNA was synthesized from 2 μg total RNA using TaqMan RT reagent kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer’s protocol. The reaction mixture (100 μl) was incubated at 25°C for 10 minutes, followed by incubation at 48°C for 30 minutes and 95°C for 5 minutes, and then cooled on ice. An aliquot of the corresponding samples diluted 1:10 was used in the real-time PCR experiments (27). The cDNA were amplified in TaqMan universal PCR master mix by the ABI Prism 7000 sequence detection system (Applied Biosystems) with primers and probe sets as shown in Table 2. Human β-actin endogenous control primers and probe set were provided by Applied Biosystems. Sequences of primers and probe were designed using primer express software provided by Applied Biosystems, and all probes were labeled with FAM/TAMRA by ABI Custom Services. The number of copies of the gene of interest in each sample was calculated from the corresponding standard curve. For each sample, the gene copy number was normalized by the value of β-actin on the same sample. Real-time PCR reactions were done in triplicate and each experiment repeated three times. The geometric mean values were calculated for each gene and condition.

Statistics. The Spearman rank test was used to analyze the correlation between gene up-regulation using quantitative RT-PCR (qRT-PCR) and microarray.

Results

Expression of hypoxia-inducible factor-1α and hypoxia-inducible factor-2α in transfected HEK293T cells. To assess the differential up-regulation of genes by HIF-1α or HIF-2α, we initially transfected HEK293T cells with plasmids encoding HIF-1α or HIF-2α. The cells were transfected under normoxic conditions because hypoxic conditions would lead to increases in endogenous HIFs. However, a potential problem with this approach is that HIFs are rapidly degraded under conditions of normoxia, so transfection of cells with a wild-type HIF plasmid may not sufficiently increase HIF levels to optimally activate HIF-sensitive genes. In previous studies using Hep3B cells, we had found that degradation was particularly an issue with HIF-1α transfection (23). To address this possibility, we utilized a mutant form of HIF-1α (HIF-1α mut) that contains mutations in proline residues at codons 402 and 564 (24, 28). This HIF-1α mut is resistant to proteosomal degradation under normoxic conditions and, thus, had advantages for assessing specific up-regulation of genes by HIF-1α.

To assess the expression of HIF-1α (or HIF-1α mut) and HIF-2α by HEK293T cells transfected with the plasmids as described above, we prepared nuclear extracts and assessed them for the presence of HIF-1α and HIF-2α by immunoblotting using antibodies to these factors (Fig. 1). These analyses were compared with the levels of HIF-1α and HIF-2α induced by hypoxia. Transfection with HIF-2α resulted in a substantial increase of the levels of this protein under normoxic conditions (Fig. 1). HIF-1α was also detected in cells transfected with HIF-1α plasmid under normoxic conditions, but levels were higher in cells transfected with HIF-1α mut. The increases in HIF-1α or HIF-2α in cells transfected under normoxic conditions were specific for the protein being transfected. HEK293T cells exposed to hypoxia had increases in both HIF-1α and HIF-2α, although the levels were less than that transfected with either factor (Fig. 1). Thus, hypoxia increased both HIF-1α and HIF-2α in nuclear extracts of these cells, and specific transfection with HIF-1α, HIF-1α mut, or HIF-2α could induce specific increases in these proteins even when the cells were cultured under normoxic conditions.

Global gene expression in HEK293T cells exposed to hypoxia or transfected with hypoxia-inducible factors. The NCI Hs-Operon V2 human oligoarray system was utilized to monitor ~20,000 human gene transcripts in cells transfected with HIF-1α,
HIF-1α mut, HIF-2α, or exposed to hypoxia. As an additional control for transfection, we examined the up-regulation of the transfected genes. Transfection with HIF-1α or HIF-1α mut resulted in a mean 21.2- or 16.1-fold increase in HIF-1α expression, respectively, but not HIF-2α. Similarly, transfection with HIF-2α resulted in a mean 13.6-fold increase in HIF-2α expression but not HIF-1α.

To assess the effects of HIF transfection or hypoxia, we focused on genes that were up-regulated by at least 3-fold under one of the conditions above as a relatively conservative cutoff. In all, 56 genes were identified that fit those criteria. These genes are shown on Fig. 2, along with the degree of up-regulation obtained under conditions of hypoxia or transfection with HIF-1α, HIF-1α mut, or HIF-2α. These genes include at least 26 that were previously reported to be up-regulated by hypoxia or HIF (12, 29–37). At the same time, we identified a number of additional genes not previously been reported to be induced by hypoxia or HIF. In nearly all cases, up-regulation by HIF-1α mut was greater to equal to that induced by HIF-1α. For subsequent analyses, we use the higher value obtained with HIF-1α or HIF-1α mut and refer to these composite results as HIF-1α/m.

We were particularly interested in analyzing the relative up-regulation of genes by HIF-1 and by HIF-2. To this end, we sorted the 56 genes into four groups based on their up-regulation with HIF-1α/m, HIF-2α, and hypoxia. In doing this, genes that were up-regulated <2-fold by a given condition were considered as not having a substantial response. The four groups were as follows: (a) genes that were up-regulated ≥2-fold by both HIF-1α/m and HIF-2α; (b) genes that were up-regulated ≥2-fold by HIF-1α/m but <2-fold by HIF-2α; (c) genes that were up-regulated ≥2-fold by HIF-2α but <2 fold by HIF-1α/m; and (d) genes that were up-regulated ≥2-fold by hypoxia but not ≥2-fold by either HIF-1α/m or HIF-2α. For one gene (gene 56, NPPB), values for neither HIF-1α nor HIF-1α mut could be determined, and this gene could not be sorted.

When sorted this way, 21 of the 56 genes (genes 1-21 in Fig. 2) were found to be up-regulated ≥2-fold by HIF-1α/m as well as HIF-2α. These genes include a number that have been previously reported to be inducible by hypoxia, including LOX, PLD2, VEGF, and ITGAV2. Fourteen genes (genes 22-35 in Fig. 2) were up-regulated ≥2-fold by HIF-1α but <2-fold by HIF-2α. It has been recently reported that hypoxia responsiveness of certain genes involved in the glycolytic pathway is mediated by HIF-1α but not HIF-2α (12). In agreement with this report, we observed that several genes of the glycolytic pathway, namely PFKFB4, ALDOC, PGK1, and LDHA, were among those found here to be up-regulated ≥2-fold by HIF-1α/m, but <2-fold by HIF-2α.

In addition to the 35 genes that were up-regulated by HIF-1α/m, we identified 10 genes (genes 36-45 in Fig. 2) that were up-regulated ≥2-fold by HIF-2α, but <2-fold by HIF-1α/m. In every case, the gene was up-regulated >3-fold by HIF-2α and the up-regulation by HIF-2α transfection was more than twice the magnitude of that found with HIF-1α/m transfection. Moreover, only three of these genes were up-regulated by ≥1.5-fold by HIF-1α/m. Taken together, these results suggested that whereas a number of HIF-sensitive cellular genes can be up-regulated by both factors, certain genes may be preferentially or even uniquely up-regulated by HIF-1α or by HIF-2α.

Of the 21 genes that were up-regulated by both HIF-1α/m and HIF-2α transfection, 15 were also up-regulated ≥2-fold by hypoxia. Likewise, of the 14 genes that were up-regulated by HIF-1α/m but not by HIF-2α, 13 were up-regulated ≥2-fold by hypoxia. Thus, of the 35 genes that were up-regulated by HIF-1α or HIF-1α mut, the vast majority were also up-regulated by hypoxia. These genes include a number that were previously described as being hypoxia and/or HIF-1α dependent. Among the 35 genes, 8 are involved with cell metabolism, 3 are oncogenes, 3 are involved in cell structure, 3 are associated with transcription, 2 are growth factors, and 2 are associated with apoptosis.

By contrast, of the 10 genes identified as being up-regulated at least 2-fold by HIF-2α but not by HIF-1α/m (genes 36-45 in Fig. 2), only 3 (GADD45B, CITED2, and PKIB) were up-regulated at least 2-fold by hypoxia. Of the remaining six genes for which hypoxia responsiveness could be determined, all had little or no up-regulation by hypoxia. It is noteworthy that the 10 genes up-regulated by HIF-2α but not HIF-1α included four genes involved in signal transduction (PKIA, PKIB, CACNA1A, and PTPRZ1) and that two of these (CACNA1A and PTPRZ1) have recently been implicated in diseases of the brain.

An additional group of 10 genes were identified by microarray that were up-regulated ≥2-fold by hypoxia, but were not up-regulated 2-fold by either HIF-1α/m or by HIF-2α transfection (genes 46-55 in Fig. 2). All but two of these genes were up-regulated at least 4-fold by hypoxia. In a number of cases, either HIF-1α/m or HIF-2α transfection induced some up-regulation of the genes, although <2-fold. Even so, it was noteworthy that the up-regulation...
by hypoxia compared with HIF transfections was relatively greater than with other genes, suggesting that other factors may be influencing the up-regulation of these genes under conditions of hypoxia.

Analysis of selected gene up-regulation by real-time reverse transcription PCR and Northern blot. We sought to utilize other techniques to analyze activation of some of the genes identified above. To this end, we studied the stimulation of a number of the genes using real-time RT-PCR and Northern blot (Fig. 3; Table 3). In particular, such studies were done on two genes up-regulated in microarray by both HIF-1α and HIF-2α (LOX and RASSF1), four genes preferentially up-regulated by HIF-1α (PPFIA4, RAB20, PFKFB4, and ALDOC), and three genes preferentially up-regulated by HIF-2α (CITED2, CACNA1A, and PTPRZ1). In addition, expression of HIF-1α was studied as a control, although not utilized in statistical calculations.

By microarray, LOX and RASSF1 were up-regulated by all three stimuli: hypoxia (6.5-fold), HIF-1α (5.0-fold), and HIF-2α (22.2-fold). Consistent with these results, we found by qRT-PCR that LOX and RASSF1 were also up-regulated by each of the three stimuli (6.0-, 5.8-, and 11.3-fold, respectively; Table 3). Moreover, the up-regulation of the other genes as assessed by qRT-PCR was strongly correlated with the results obtained by microarray (r = 0.92, P < 0.0001). There was also a strong correlation between the two techniques when up-regulation induced by hypoxia (r = 0.86) and HIF-2α (r = 0.96) were looked at separately, although less when HIF-1α up-regulation was examined separately (r = 0.57).

RAB20, PFKFB4, BNP3, RASSF1, LOX, ALDOC, and HIF-1α RNA levels were also analyzed by Northern blot (Fig. 3). h-actin serves as loading control.

Figure 2. Genes up-regulated at least 3-fold in HEK293T cells exposed to hypoxia (Hypo) or transfected with HIF 1α, HIF-1α mut, or HIF-2α. Results shown are expressed as the antilog of the mean of three log2-transformed ratios of hypoxia or transfection versus normoxia was for the nonflagged results from the triplicate determinations. The genes are divided into groups based on their up-regulation with HIF-1α or HIF 1α mut, HIF-2α, and hypoxia as described in the text. Specific information on each gene can be found by looking up the feature ID on http://nciarray.nci.nih.gov/. A number of these genes (marked with asterisks) have been described previously as being responsive to hypoxia and/or HIF (4, 12, 29–37).
Northern blot. HEK293T cells were exposed to normoxia (N) or hypoxia (H) for 16 hours, or transfected with HIF-1α, HIF-1α/m, or HIF-2α plasmids under normoxic condition. HIF-1α preferentially up-regulated BNIP3, RAB20, RASSF1, HIF-2α preferentially up-regulates LOX. HIF-1α and β-actin are shown as controls.

HIF-1α/m as assessed by Northern blot seemed even greater than that assessed by microarray. Thus, in the cases where it was assessed, real-time qRT-PCR and Northern blot analysis confirmed the gene expression patterns as assessed by microarray.

Discussion

A number of previous studies have provided evidence that many of the cellular effects of hypoxia are mediated by increases in the levels of HIF-1α and/or HIF-2α. In this study, we found that a number of genes were up-regulated both by overexpression of HIF-1α/m or HIF-2α in the human embryonic kidney cell line HEK293T. However, certain genes were preferentially or even specifically activated by HIF-1α/m and certain other genes by HIF-2α. Ten of the genes up-regulated by HIF-1α/m and/or HIF-2α were also examined using qRT-PCR and six also by Northern blot analysis, and the results paralleled the gene activation observed with microarray. Taken together, these results suggest that whereas a number of genes are activated similarly by both factors, other genes can be preferentially activated by one or the other in a transfected cell line model.

Most of the genes that were up-regulated by HIF-1α/m in this system were also up-regulated by hypoxia. These results are consistent with a key role of HIF-1α in mediating the genetic response to hypoxia in these cells, although it should be stressed that up-regulation by HIF-1α/m (or HIF-2α) in transfected cells does not, by itself, prove a role of the factor in mediating the response to hypoxia. Twenty-one of these HIF-1α/m-responsive genes were also activated ≥2-fold by HIF-2α, whereas an additional 14 genes seemed to be preferentially or selectively up-regulated by HIF-1α/m. The genes preferentially activated by HIF-1α/m included several involved in glycolysis, consistent with a previous report by Hu et al. (12), who reported that up-regulation of certain glycolytic genes (including PGK1 and LDHA) by hypoxia is exclusively mediated by HIF-1α. The results here extend a preferential or exclusive role of HIF-1α to other glycolytic genes, including ALDOC and PFKB4. At the same time, we also confirmed the finding that CXCR4 is up-regulated by HIF-1α (37) and identified other genes preferentially up-regulated by HIF-1α, including two involved with apoptosis (BNIP3L and BNIP3) and one involved with signal transduction (PPEIA4).

In the current study, 10 genes were found to be up-regulated ≥2-fold by HIF-2α but not by HIF-1α. Three of these genes (GADD45B, CITED2, and PKIB) were also up-regulated by hypoxia in HEK293T cells. It should be noted, however, that these three genes were weakly up-regulated by HIF-1α transfection, albeit <2-fold, suggesting that the genes were preferentially, rather than only, responsive to HIF-2α. In the case of CITED2, the preferential up-regulation by HIF-2α was confirmed by qRT-PCR. The preferential activation of this gene is of interest given recent reports that it is a negative regulator of HIF-1α activity (38, 39). In addition to these three genes that were up-regulated by HIF-2α and by hypoxia, six additional

Table 3. Comparison of gene up-regulation as assessed by qRT-PCR and microarray

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NOTE: Data shows fold increase under each condition.
genes were found to be preferentially or selectively up-regulated by HIF-2α but not by hypoxia in HEK293T cells. It is possible that this difference is because of the substantially greater expression of HIF-2α in transfected HEK293T cells compared with cells exposed to hypoxia, although we could not assess this quantitatively. However, even in the case of Hep 3B cells, in which there is relatively greater expression of HIF-2α with hypoxia (23), these genes did not seem to be up-regulated by hypoxia (results not shown). These results suggest that the relationship between HIF-2α gene activation and the cellular response to hypoxia is complex.

Several papers suggest that HIF-1α and HIF-2α may play different roles in different cell types and that HIF-2α is especially important in endothelial cells. Also, questions have been raised as to the role of HIF-2α in hypoxia. Park et al. (40), for example, have reported that at least in mouse embryo fibroblasts exposed to hypoxia, HIF-2α remains localized to the cytoplasm and does not mediate transcription in the nucleus. By contrast, we were able to identify HIF-2α in nuclear extracts of HEK293T cells exposed to hypoxia (Fig. 1), suggesting that HIF-2α has the potential to mediate a response to hypoxia in these cells. Hu et al. (12) have provided evidence that in a renal carcinoma cell line lacking HIF-1α, the response to hypoxia could be mediated by HIF-2α. The experiments here show that under conditions of gene overexpression, HIF-2α has the potential to up-regulate a group of genes in the HEK293T immortalized cell line. The studies do not, in themselves, prove that physiologic activation of HIF-2 (through hypoxia and/or other mechanisms) can up-regulate these genes, and it will be important to address this issue as well as the broad question of the role of HIF-2. Studies of HIF-2 may be particularly important in understanding the tumors that develop in patients with VHL, as there is recent evidence suggesting that the tumorigenic phenotype in such patients is more related to HIF-2 than HIF-1 overexpression (20, 21).

It has recently been reported that the gene for vascular endothelial growth factor receptor-2 (Flk-1) was induced by HIF-2α, but not by HIF-1α, and that this effect involved a cooperative interaction with Ets-1 (41). Also, Aprelikova et al. (42) have recently provided evidence that the gene PHD3 was preferentially induced by HIF-2α as opposed to HIF-1α in two different cell lines. Whereas our microarray chips did not include sequences for either of these genes, it was noteworthy that several genes were preferentially up-regulated by HIF-2α in our system including four involved in signal transduction (PKIA, PKIB, CACNA1A, and PTPRZ1). Interestingly, there is recent evidence that two of these genes may play an important role in diseases of the brain. CACNA1A forms voltage-dependent Ca2+ channels in neuronal tissue and mutations in this gene are associated with hemiplegic migraines, familial migraines, and episodic ataxia (43–45). Protein tyrosine phosphatase receptor type Z (PTPRZ1) expression is limited to the central nervous system, and this protein has recently been identified as important in the recovery from demyelinating lesions (46). It has been hypothesized that this gene may play a role in the pathogenesis of multiple sclerosis. The findings here raise the question of whether HIF-2 may play a role in the central nervous system gene regulation independent of hypoxia and may possibly lead to insights in the pathogenesis and treatment of these diseases.

The observation that certain genes are selectively or preferentially up-regulated by HIF-1α or by HIF-2α raises the question as to the basis for this factor selectivity. Earlier studies have indicated that both HIF-1 and HIF-2 can bind to the same hypoxic response elements (5, 11, 14, 19). Hypoxic response elements generally consist of a HIF-binding sequence and a HIF ancillary sequence, and there is a degree of variation in both among different genes. It is possible that certain hypoxic response elements preferentially bind to HIF-1 or HIF-2. This would be similar to the situation with hormone response elements in which the particular sequence may lead to binding to either androgens or glucocorticoids alone or to both androgens and glucocorticoids (47, 48). The recent finding that a cooperative interaction between HIF-2α and Ets-1 is important in the activation of Flk-1 may provide one mechanism for HIF-2α specificity (41). Additional studies will be needed to investigate whether a similar mechanism is involved in other genes preferentially up-regulated by HIF-1α or HIF-2α.

Finally, it was noteworthy that 10 genes were identified in these studies that were up-regulated by hypoxia >3-fold but were not up-regulated by 2-fold by either HIF-1α (or HIF-1α mut) or by HIF-2α. In most cases, these genes were up-regulated by ≥1.5-fold by HIF-1α mut (and in some cases also by HIF-2α), suggesting that this is a relative effect and that hypoxia responsiveness of these genes may be mediated, in part, through HIF-1. These results raise the question as to whether yet another factor was mediating or contributing to the hypoxia responsiveness in these genes as has been described for certain HIF-responsive genes (49–51). In this regard, there is some evidence suggesting that cyclic AMP response element–binding protein and nuclear factor-κB may mediate certain effects of hypoxia (50, 51). Alternatively, it is possible that activation of these genes requires both HIF-1 and HIF-2 up-regulation.

In summary, the results here provide evidence of several new genes that are responsive to HIFs and that genes may vary in their relative sensitivity to HIF-1 and HIF-2, at least in the cell line utilized. Additional studies will be needed to clarify the responsive-ness of these genes to hypoxia, HIF-1, and HIF-2 using other cell lines and other experimental systems. The results add to previous studies in discerning the biological significance of these two separate factors and may serve to stimulate further studies aimed at understanding the mechanisms responsible for their selectivity and its biological significance. An understanding of these results may lead to strategies to selectively inhibit certain responses to hypoxia in a therapeutically advantageous way.

Acknowledgments

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References

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