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Hypoxia-inducible Factor-1-mediated Expression of the 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (*PFKFB3*) Gene:

ITS POSSIBLE ROLE IN THE WARBURG EFFECT*

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Abstract

One of the key mediators of the hypoxic response in animal cells is the hypoxia-inducible transcription factor-1 (HIF-1) complex, in which the α-subunit is highly susceptible to oxygen-dependent degradation. The hypoxic response is manifested in many pathophysiological processes such as tumor growth and metastasis. During hypoxia, cells shift to a primarily glycolytic metabolic mode for their energetic needs. This is also manifested in the HIF-1-dependent up-regulation of many glycolytic genes. Paradoxically, tumor cells growing under conditions of normal oxygen tension also show elevated glycolytic rates that correlate with the increased expression of glycolytic enzymes and glucose transporters (the Warburg effect). A key regulator of glycolytic flux is the relatively recently discovered fructose-2,6-bisphosphate (F-2,6-P2), an allosteric activator of 6-phosphofructo-1-kinase (PFK-1). Steady state levels of F-2,6-P2 are maintained by the bifunctional enzyme PFK-2/F2,6-Bpase, which has both kinase and phosphatase activities. Herein, we show that one isozyme, PFKFB3, is highly induced by hypoxia and the hypoxia mimics cobalt and desferrioxamine. This induction could be replicated by the use of an inhibitor of the prolyl hydroxylase enzymes responsible for the von Hippel Lindau (VHL)-dependent destabilization and tagging of HIF-1α. The absolute dependence of the *PFKFB3* gene on HIF-1 was confirmed by its overexpression in VHL-deficient cells and by the lack of hypoxic induction in mouse embryonic fibroblasts conditionally nullizygous for HIF-1α.

The rate of glucose utilization via the glycolytic pathway is highly regulated and depends upon the energetic and metabolic needs of the cell. It is coordinated with other pathways of energy generation and utilization, notably gluconeogenesis, the pentose phosphate pathway, and the citric acid cycle. Fructose-2,6-bisphosphate (F-2,6-P2)[1] is considered to be the

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[1]The abbreviations used are: F-2,6-P2, fructose-2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase; PFKFB3, 6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase-3; HIF-1α, hypoxia-inducible factor-1α; VHL, von Hippel Lindau; Glut-1, glucose transporter-1; VEGF, vascular endothelial growth factor; HRE, hypoxia-responsive element.

major regulator controlling carbon flux through glycolysis. F-2,6-P2 is an allosteric activator of 6-phosphofructo-1-kinase (PFK-1), the key regulatory enzyme in glycolysis as well as an inhibitor of frucrose-1,6-bisphosphatase (1–3). The synthesis and degradation of F-2,6-P2 depends upon a single enzyme, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK-2/F-2,6-BPase), which has both kinase and phosphatase activities. This bifunctional enzyme is regulated by phosphorylation and dephosphorylation that are dependent upon intracellular cAMP levels (4). Furthermore, PFK-2/F-2,6-BPase synthesis can be induced by mitogens, growth factors, and inflammatory cytokines, implicating its role in setting the glycolytic rate under multiple physiologic and pathologic conditions (5).

Four different genes coding different isozymes (*PFKFB1–4*) have been identified to date (6–9). These isoenzymes differ not only in their tissue distribution but also in their kinetic and regulatory properties. The PFKFB3 isozyme has the highest kinase:phosphatase activity ratio and thus maintains elevated F-2,6-P2 levels, which in turn sustains high glycolytic rates (5, 10). Significantly, this isoform is constitutively expressed in several human cancer cell lines having high proliferative rates that require the elevated activity of the enzyme for the synthesis of 5-phosphoribosyl-1-pyrophosphate, a precursor for purines and pyrimidines (5, 11, 12). Thus, this could serve as an explanation for the high glycolytic rates present in transformed cells even under normal oxygen tension (the Warburg effect).

Hypoxia is a potent inducer of gene expression. It is also an important component of many pathophysiological processes including tumor growth and metastasis (13, 14). In hypoxic conditions, as oxidative phosphorylation is impaired, cells turn to glycolysis to meet their energetic demands (the Pasteur effect). During the adaptive response to hypoxia, the expression of genes encoding several of the glycolytic enzymes and glucose transporters is increased (15, 16). In animals, the hypoxiainducible transcription factor-1 (HIF-1) complex mediates the activation of these genes. HIF-1 is a heterodimeric protein complex composed of two subunits: a constitutively expressed β-subunit, and an α-subunit for which expression and activity are controlled by intracellular oxygen concentration (reviewed in Refs. 17 and 18). During normoxia, HIF-1α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation (19–21). The enzymatic hydroxylation of proline 564 of HIF-1α controls the turnover of the protein by tagging it for interaction with the von Hippel Lindau (VHL) protein (22–24). The VHL protein forms a multiprotein complex that contains, at a minimum, elongins B and C, Cul2, and Rbx, and acts as the ubiquitin ligase that targets HIF-1α for degradation. The effect of hypoxia on Pro-564 hydroxylation can be mimicked by transition metals like cobalt, iron chelators and by inhibitors of the prolyl hydroxylase enzymes (22, 23).

Despite its importance in regulating glycolysis and gluconeogenesis, the role of PFK-2/F-2,6-BPase enzyme in the hypoxic response pathway in mammals has not been characterized. We report here that hypoxia, cobalt, and iron chelators produce a significant induction of PFKFB3 mRNA in several human and mouse cell lines. Furthermore, by utilizing conditional knockout cell lines of the *HIF-1*α gene, we demonstrate that the hypoxia inducibility of this gene is dependent on the presence of an active HIF-1 complex.

EXPERIMENTAL PROCEDURES

Materials

Cobalt chloride and desferrioxamine were purchased from Sigma. Dimethyloxalylglycine was a gift of Peter Ratcliffe (Oxford, UK). Fetal calf serum was obtained from HyClone (Logan, UT). [^{32}P]UTP (800 Cu/mmol) was from PerkinElmer Life Sciences. T3 and T7 RNA polymerases, RNase inhibitor, and DNase I (Rnase free) where from Roche Molecular Biochemicals.

Cell Lines and Culture Conditions

Human hepatoma Hep-3B cells and RPE (human retinal pigment epithelial) cells were grown in minimum essential medium supplemented with pyruvate, 10–15% fetal bovine serum, penicillin, and streptomycin. Mouse embryonic fibroblasts (wild-type and HIF-1α-deficient cell lines) were obtained from R. S. Johnson (University of California, San Diego, La Jolla, CA) (14) and grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Western blot analysis of this conditionally nullizygous fibroblasts for HIF-1α has shown the absence of HIF-1α mRNA and protein (14). Mitochondrial DNA-less (143B206) ρ° cells and their parental osteosarcoma cell line (143B) were described previously (25). The pVHL-deficient 786-0 renal carcinoma cells and a stable transfectant cell line expressing wild-type pVHL were provided by Nikolai Kley (Bristol-Myers Squibb).

For hypoxic treatment, the culture plates were placed for 6 h in a modular incubator chamber (Billup-Rothenburg, Forma Scientific, Marietta, OH) and flushed with a gas mixture containing 0.5% oxygen, 5% carbon dioxide, and 94% nitrogen. This low oxygen concentration was chosen to obtain maximal HIF-1α hypoxic stimulation, as reported by Jiang *et al.* (42).

RNA Isolation

Total RNA was extracted from cultured cell lines using the acid guanidinium-phenol-chloroform extraction method described by Chomczynski and Sacchi (26). Cells were extracted with 2 ml of guanidine isothiocyanate solution (UltraPure) (4 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 0.1 M 2-mercaptoethanol) directly in the plates. Sequentially, 0.2 ml of 2 M sodium acetate, pH 4.0, 2 ml of phenol (water-saturated), and 0.4 ml of a chloroform-isoamyl alcohol mixture (49:1) were added to cell lysate with thorough mixing after the addition of each reagent. RNA was precipitated with an equal volume of 2-propanol. RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water.

Ribonuclease Protection Assay

The plasmid for synthesis of human 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) probe for ribonuclease protection assay was created by synthesis of a cDNA using total RNA from Hep-3B cells and oligo(dT) followed by cloning. PFKFB3 cDNA was amplified using forward primer (5′-GGCCGCATCGGGGGCGACTC-3′) and reverse primer (5′-TTGCGTCTCAGCTCAGGGAC-3′). These oligonucleotides correspond to

nucleotide sequences 901–920 and 2250–2231 of the human PFKFB3 cDNA, respectively (GenBank™ accession number NM004566) (9). The PCR fragment was cloned into plasmid pCR II-TOPO using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). An *Eco*RI-*Eco*47III fragment from this plasmid corresponding to nucleotide sequences 901–1218 of the human PFKFB3 cDNA was recloned into pBluescript II KS^+ (Stratagene, La Jolla, CA) using *Eco*RI and *Eco*RV sites. Following digestion with *Xba*I, this plasmid was utilized to create a 419-base radiolabeled antisense probe for ribonuclease protection assay. This probe recognizes a common region expressed in both the inducible and ubiquitous PFKFB3 isoforms. A similar procedure was utilized for the synthesis of a mouse probe using mouse spleen total RNA as template. The forward primer (5′-GGCAAGATTGGGGGCGACTC-3′) and reverse primer (5′GGCTCCAGGCGTTGGACAAG-3′) correspond to nucleotide sequences 1112–1131 and 1440–1421 of the mouse PFKFB3 cDNA, respectively (GenBank™ accession number AF294617). The final PCR fragment was cloned into plasmid pCR II-TOPO and following digestion with *Bam*HI was utilized for the mouse PFKFB3 probe generation. The human Glut-1 probe was created by synthesis of a cDNA using total RNA from HeLa cells and oligo(dT) followed by cloning. Glut-1 cDNA was amplified using forward primer (5′-CCATGGAGCCCAGCAGCAAG-3′) and reverse primer (5′-ATGGCAGCTGGACGTGGACC-3′). These oligonucleotides correspond to nucleotide sequences 178–197 and 1390–1371 of the human Glut-1 cDNA, respectively (GenBank™ accession number NM006516). Following cloning and insertion into pBluescript II KS^+, the plasmid was digested with *Xba*I to create a 478-base radiolabeled antisense probe. The plasmid for synthesis of mouse Glut-1 probe for ribonuclease protection assays was created by synthesis of a cDNA using total RNA from mouse lungs and oligo(dT) followed by cloning. Glut-1 cDNA was amplified using forward primer (5′-CCATGGAGCCCAGCAGCAAG-3′) and reverse primer (5′-CACACCGATGATGAAGCGGC-3′). These oligonucleotides correspond to nucleotide sequences 188–207 and 582–563 of the mouse Glut-1 cDNA, respectively (GenBank™ accession number M23384). PCR fragment of Glut-1 cDNA was cloned into plasmid pCR II-TOPO. This plasmid was digested with *Hin*dIII to make a 443-base radiolabeled antisense probe. The probe utilized for the human VEGF ribonuclease protection assay has been already described (27). The plasmid for synthesis of mouse 18 S probe was created by synthesis of a cDNA using mouse lung total RNA and reverse primer followed by cloning of the cDNA, essentially as described above. The oligonucleotides utilized correspond to nucleotide sequences 233–251 and 860–843 of mouse 18 S rRNA, respectively (GenBank™ accession number X00686). A *Pst*I-*Xba*I fragment was recloned into pBluescript II SK^+, and following digestion with *Xba*I, this plasmid was utilized to generate a 435-base radiolabeled antisense probe that was used to analyze mouse and human total RNA.

All constructs were verified by sequencing the inserts and found to be 100% identical to the published sequences. Synthesis of radiolabeled probes was performed following Roche protocols using T7 or T3 RNA polymerase (Roche Molecular Biochemicals) and [α^{32}P]UTP. For ribonuclease protection assays, water solutions of total RNA were dried under vacuum and dissolved in 25 μl of 80% formamide hybridization buffer containing labeled probes. Samples were preincubated for 5 min at 85 °C and then incubated for 16 h at 45 °C as described previously (27). The extracted, protected probe fragments were run on a

6% polyacrylamide sequencing gel in 1× Tris-borate-EDTA buffer for 2 h at 50 mA. The gel was then dried and exposed to x-ray film (Hyperfilm MP, Amersham Biosciences, Inc.) at −70 °C. Expression of mRNA was quantified using storage phosphor technology (Molecular Dynamics, Sunnyvale, CA). Intensity of each mRNA band was normalized for 18 S ribosomal RNA level.

Electrophoretic Gel Shift Assays and Western Blots

Gel shift assays where performed as already described (25) using nuclear extracts from normoxic and hypoxic cells. The 18-mer radiolabeled probe used for the gel shift assay contained the HRE consensus sequence from the erythropoietin gene. Western blots utilized total cell extracts as described (25). Anti-HIF-2α antibody was from Novus Biologicals (Littleton, CO), and antiVHL antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). For supershift assays, anti-HIF-1α from Novus Biologicals was utilized.

RESULTS

Induction of PFKFB3 by Hypoxia, Cobalt, and Desferrioxamine

To examine the effects of hypoxia, cobalt and desferrioxamine on *PFKFB3* gene expression, mRNA levels were measured by RNase protection assays. As shown in Fig. 1*a*, hypoxia, cobalt, and desferrioxamine greatly stimulated PFKFB3 and Glut-1 mRNA expression in Hep-3B cells. Furthermore, as shown in Fig. 1*b*, mRNA accumulation is visible by 2 h following treatment with desferrioxamine and is maximal at 18 h. A similar pattern of induction is seen for Glut-1 mRNA. The effect of hypoxia and desferrioxamine was also tested in ρ° cells that lack mitochondrial DNA and have no functional respiratory electron transport (25). These cells depend exclusively on glycolysis for their energetic needs, and there have been conflicting reports on their response to hypoxia as both normal and abnormal (25, 28, 29). As shown in Fig. 1*c*, ρ° cells, as well as their parental control cells, have a normal induction of the *PFKFB3* gene in response to hypoxia and desferrioxamine. Unexpectedly, no induction of PFKFB3 was observed in HeLa cells (not shown).

Effect of HIF-1 Complex on PFKFB3 Expression

To test the role of HIF-1 in the hypoxic response of the *PFKFB3* gene, we utilized a mouse fibroblast cell line with a conditional deletion of the *HIF-1*α gene (14). Wild-type cells and HIF-1α negative cells were exposed to hypoxia for 6 h, and PFKFB3 was analyzed using the RNase protection assay. As shown in Fig. 2*a*, although HIF-1α positive cells have a substantial induction of PFKFB3 mRNA in hypoxia, no changes were seen in the HIF-1α negative cells. Similar effects were observed for the *Glut-1* and *VEGF* genes, for which the response to hypoxia is known to be dependent on HIF-1. The gel shift in Fig. 2*b* shows the hypoxic induction of the HIF-1 complex in the control HIF-1α (+), whereas no such complex is seen in the negative cells. The presence of HIF-1α in the hypoxic HIF-1α (+) cells was confirmed by supershift assay (Fig. 2*b*).

Effect of Prolyl Hydroxylase Inhibition on PFKBF3 mRNA Expression

Oxygen sensing is mediated by an oxygen-dependent hydroxylation of Pro-564 in the ODD (oxygen-dependent degradation) domain of HIF-1α protein. This reaction is mediated by

specific iron-dependent prolyl hydroxylases, which utilize oxoglutarate as a co-substrate (30, 31). Inhibition of these enzymes can induce HIF-1α under normoxic conditions (23). We utilized dimethyloxalylglycine, a cell-permeable competitive inhibitor of oxoglutarate, to examine the effects of enzyme inhibition on PFKBF3 mRNA expression. As shown in Fig. 3*a*, dimethyloxalylglycine treatment for 6 h results in the accumulation of PFKFB3 and Glut-1 mRNA to a degree equivalent to that produced by hypoxic stimulation in two different cell lines. As expected, dimethyloxalylglycine stimulates HIF-1α expression as shown by Western blot analysis in Fig. 3*b*.

Role of VHL in PFKFB3 Expression

HIF-1α hydroxylation at Pro-564, and possibly at others residues (32), facilitates its interaction with pVHL, which ultimately targets it for ubiquitination and degradation by the proteasomal system. Cells deficient in pVHL have been shown to over-express HIF-α proteins and up-regulate HIF-1-dependent genes such as VEGF and Glut-1 (33). To test the role of pVHL on PFKFB3 mRNA expression, we utilized the pVHL-deficient 786-0 renal carcinoma cell line. These cells contain a missense VHL mutation and fail to produce a functional VHL protein. As shown in Fig. 4*a*, VHL-deficient (−) cells overexpress PFKFB3, Glut-1, and VEGF messengers. The expression of these genes is attenuated in 786-0 cells with stable transfection of a plasmid expressing the wild form of pVHL (+). Fig. 4*b* shows the increased levels of pHIF-2α in the VHL (−) cells corresponding to the absence of pVHL.

DISCUSSION

The hypoxic response of an organism is manifested both at the systemic and at the cellular level. When oxygen becomes limited, cells switch from oxidative phosphorylation to anaerobic glycolysis. Fructuose-2,6-biphosphate is the most potent activator of glycolysis and exerts control over the rate of glucose utilization (1–4). It allosterically activates PFK-1 and inhibits the gluconeogenic enzyme fructuose-1,6-bisphosphatase. PFK-2/F-2,6-BPase controls the levels of Fru-(2,6)-P2 by catalyzing its synthesis and degradation. Among the PFK-2/F-2,6-BPase isoforms of mammalian origin, PFKFB3 has the highest kinase: phosphatase activity. It is also the isoform most highly expressed in transformed cells, suggesting that it may contribute to the high glycolytic rate observed in tumors (5, 10).

Although several studies have demonstrated that the genes coding for some of the enzymes of the glycolytic pathway are activated during hypoxia, the response of the PFK-2/F-2,6-BPase gene has not been characterized. Our studies with the PFKFB3 isoform demonstrate that exposure to hypoxia produced a significant increase in its mRNA in several cell lines. However, HeLa cells, which have a high basal level of expression, did not show responses to hypoxia. The response to hypoxia was mimicked by exposure to the iron chelator desferrioxamine and the transition metal cobalt. Furthermore, the induction of the *PFKFB3* gene was not affected by the absence of oxidative phosphorylation, as shown by the normal response in ρ° cells. These cells, which lack mitochondrial DNA and depend exclusively on glycolysis, have yielded conflicting results with respect to their responses to hypoxia (25, 28, 29). The results presented here confirm that gene activation in response to hypoxia is independent of the presence of an active mitochondrial respiratory chain. The role of HIF-1

was investigated using cells deficient in the HIF-1α subunit. It has been shown that the HIF-1α (−/−) cells have decreased growth rate under hypoxic conditions as well as impaired glycolytic response to hypoxia (Pasteur effect), manifested by lower ATP levels, decreased lactic acid production, and decreased acidosis (34). The response to hypoxia of the *PFKFB3* and the *Glut-1* genes was completely suppressed in (−/−) cells, indicating an absolute requirement of a functional HIF-1 complex.

Under normoxic conditions, pHIF-1α is ubiquitinated and rapidly degraded by the proteasome system (19–21). The mechanism by which oxygen regulates the half-life of pHIF-1α involves the hydroxylation of Pro564 (and possibly other residues) by specific prolyl hydroxylase enzymes (22, 23). Following hydroxylation, HIF-1α interacts with pVHL, which acts as a ubiquitin ligase, thus targeting it for degradation. We examined the role of VHL and prolyl hydroxylation in the up-regulation of PFKFB3 using pVHL-deficient cells and competitive inhibitors of the prolyl hydroxylase enzymes. VHL-deficient cells overexpress several of the HIF-1-dependent genes as a result of their inability to degrade HIF-α proteins (33). The VHL-deficient 786-0 cells show elevated basal levels of PFKFB3, VEGF, and Glut-1 mRNAs that could be partially, but significantly, suppressed by the expression of a functional VHL protein. HIF-prolyl hydroxylases are a group of recently described enzymes that post-translationally modify HIF-1α in an oxygen concentration-dependent manner (30, 31). The structural analysis and catalytic mechanisms of members of oxygenases of this type have demonstrated the requirement for iron and oxoglutarate for their activity. Jaakkola and co-workers (23) showed that the enzymatic activity of the HIF-prolyl hydroxylases could be inhibited by 2-oxoglutarate analogs. The present studies demonstrate that *N*-oxalylglycine results in a significant up-regulation of PFKFB3 and Glut-1 mRNA expression.

The results discussed above suggest that the HIF complex mediates the activation of the *PFKFB3* gene. The structure of the *PFKFB3* gene has been well characterized. The gene spans more than 26 kb containing at least 16 exons (36). This accounts for the reported ubiquituous and inducible forms generated by alternative splicing of exon 15. Our probe, which included sequences from nucleotides 901–1218, could not differentiate between these two isoforms. Computer data base analysis of the promoter region of the PFKFB3 gene revealed a TATA box and potential binding sites for several transcription factors. Several G + C-rich regions present in the proximal 5′-end are consistent with putative motifs for the binding of Sp1 and AP-2 factors (35, 36). Potential HRE sequences (5′-RCGTG-3′) are found at several positions in the 5′ flanking region of the gene. One of them, at position −103 to −108 from the transcription start site, conforms to the consensus HRE found in most of the glycolytic enzymes and Glut-1 (18). However, the exact HIF-1 binding site involved in the hypoxic regulation of the *PFKFB3* gene has not been yet determined. In addition, a possible effect of hypoxia on the stability of PFKFB3 mRNA has not been studied.

Phosphofructuokinase is the key enzyme that regulates glycolysis in mammalian cells. It is allosterically activated by ADP and AMP and inhibited by ATP. It is believed that F-(2,6)-P2 is the most potent activator of the enzyme. Furthermore, the levels of F-(2,6)-P2 are controlled by a balance of the kinase and phosphatase activities of PFK-2/F-2,6-BPase, which vary in different cell types and are modulated under various metabolic conditions (3).

Our demonstration that hypoxia increases the expression of the PFKFB3 isoform is compatible with an adaptive response that enhances glycolysis during periods of oxygen deficiency. Tumor growth and expansion depends on their ability to generate new blood vessels (angiogenesis) and their capacity to adapt their metabolism to the hypoxic conditions created by their own growth. The activation of the HIF-1 complex is a critical response in tumor cells. It results in the enhanced transcription of a variety of genes that increase oxygen and nutrient supply (17, 18). These adaptations, as shown here, are likely to include PFKFB3, with activation of the glycolytic pathway. It should be noted that hypoxia is not the only condition that stabilizes and activates HIF-1α. Certain hormones and growth factors, including insulin, angiotensin II, and platelet-derived growth factor, and cytokines such as interleukin-1 and tumor necrosis factor can also lead to HIF-1α stabilization (37, 38). In tumors, HIF-1α is often expressed constitutively, even under normoxic conditions as is the case in VHL mutations, or as a consequence of other oncogenic activities (39–41). It is tempting to speculate that in those cases PFKFB3 may be over-expressed, thus providing an explanation for the Warburg effect, which is so often found in tumor cells (43).

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REFERENCES

1. Kawaguchi T, Veech RL, Uyeda K. J. Biol. Chem. 2001; 276:28554–28561. [PubMed: 11373280]
2. Hue L, Rousseau GG. Adv. Enzyme Regul. 1993; 33:97–110. [PubMed: 8395137]
3. Okar DA, Lange AJ. Biofactors. 1999; 10:1–14. [PubMed: 10475585]
4. Pilkis SJ, Claus TH, Kurland IJ, Lange AJ. Annu. Rev. Biochem. 1995; 64:799–835. [PubMed: 7574501]
5. Chesney J, Mitchell R, Benigni F, Bacher M, Spiegel L, Al-Abed Y, Han JH, Metz C, Bucala R. Proc. Natl. Acad. Sci. U. S. A. 1999; 96:3047–3052. [PubMed: 10077634]
6. Algaier J, Uyeda K. Biochem. Biophys. Res. Commun. 1988; 153:328–333. [PubMed: 2837207]
7. Heine-Suner D, Diaz-Guillen MA, Lange AJ, Rodriguez de Cordoba S. Eur. J. Biochem. 1998; 254:103–110. [PubMed: 9652401]
8. Sakai A, Kato M, Fukasawa M, Ishiguro M, Furuya E, Sakakibara R. J. Biochem. 1996; 119:506–511. [PubMed: 8830046]
9. Manzano A, Rosa JL, Ventura F, Perez JX, Nadal M, Estivill X, Ambrosio S, Gil J, Bartrons R. Cytogenet. Cell Genet. 1998; 83:214–217. [PubMed: 10072580]
10. Sakakibara R, Kato M, Okamura N, Nakagawa T, Komada Y, Tominaga N, Shimojo M, Fukasawa M. J. Biochem. 1997; 122:122–128. [PubMed: 9276680]
11. Boada J, Roig T, Perez X, Gamez A, Bartrons R, Cascante M, Bermudez J. FEBS Lett. 2000; 480:261–264. [PubMed: 11034341]
12. Hirata T, Watanabe M, Miura S, Ijichi K, Fukasawa M, Sakakibara R. Biosci. Biotechnol. Biochem. 2000; 64:2047–2052. [PubMed: 11129574]
13. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ. Proc. Natl. Acad. Sci. U. SA. 1997; 94:8104–8109.
14. Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS. Cancer Res. 2000; 60:4010–4015. [PubMed: 10945599]

15. Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ. Proc. Natl. Acad. Sci. U. S. A. 1994; 91:6496–6500. [PubMed: 8022811]
16. Semenza GL, Jiang B-H, Leung SW, Passantino R, Concordet J-P, Maire P, Giallongo A. J. Biol. Chem. 1996; 271:32529–32537. [PubMed: 8955077]
17. Wenger, RH.; Gassmann, M. Environmental Stress and Gene Regulation. Storey, KB., editor. Oxford: BIOS Scientific Publishers Ltd; 1999. p. 25-45.
18. Semenza GL. Genes Dev. 2000; 14:1983–1991. [PubMed: 10950862]
19. Huang LE, Gu J, Schau M, Bunn HF. Proc. Natl. Acad. Sci. U. S. A. 1998; 95:7987–7992. [PubMed: 9653127]
20. Salceda S, Caro J. J. Biol. Chem. 1997; 272:22642–22647. [PubMed: 9278421]
21. Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. J. Biol. Chem. 1999; 274:6519–6525. [PubMed: 10037745]
22. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. Science. 2001; 292:464–468. [PubMed: 11292862]
23. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreti HF, Mukherju M, Schofield CJ, Maxwell PhH, Pugh CW, Ratcliffe PJ. Science. 2001; 292:468–472. [PubMed: 11292861]
24. Yu F, White SB, Zhao Q, Lee FS. Proc. Natl. Acad. Sci. U. S. A. 2001; 98:9630–9636. [PubMed: 11504942]
25. Srinivas V, Leshchinsky I, Sang N, King M, Minchenko A, Caro J. J. Biol. Chem. 2001; 276:21995–21998. [PubMed: 11342528]
26. Chomczynski P, Sacchi N. Anal. Biochem. 1987; 162:156–159. [PubMed: 2440339]
27. Minchenko A, Bauer T, Salceda S, Caro J. Lab. Invest. 1994; 71:374–379. [PubMed: 7933988]
28. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. J. Biol. Chem. 2000; 275:25130–25138. [PubMed: 10833514]
29. Vaux EC, Metzen E, Yeats KM, Ratcliffe PJ. Blood. 2001; 98:296–302. [PubMed: 11435296]
30. Epstein ACR, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian Y-M, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. Cell. 2001; 107:43–54. [PubMed: 11595184]
31. Bruick RK, McKnight SL. Science. 2001; 294:1337–1340. [PubMed: 11598268]
32. Masson N, William C, Maxwell PH, C W, Pugh CW, Ratcliffe PJ. EMBO J. 2001; 20:5197–5206. [PubMed: 11566883]
33. Maxwell PH, Wiesner MD, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. Nature. 1999; 399:271–275. [PubMed: 10353251]
34. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K, Johnson RS. Mol. Cell. Biol. 2001; 21:3436–3444. [PubMed: 11313469]
35. Fukasawa M, Takayama E, Shinoniya N, Okumura A, Rocutanda M, Yamamoto N, Sakakibara R. Biochem. Biophys. Res. Commun. 2000; 267:703–708. [PubMed: 10673355]
36. Navarro-Sabate A, Manzano A, Riera L, Rosa JL, Ventura F, Bartrons R. Gene. 2001; 264:131–138. [PubMed: 11245987]
37. Richard DE, Berra E, Pouysségur J. J. Biol. Chem. 2000; 275:26765–26771. [PubMed: 10837481]
38. Thornton R, Lane P, Borghaei R, Paese E, Caro J, Mochan E. Biochem. J. 2000; 350:307–312. [PubMed: 10926858]
39. Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. Am. J. Pathol. 2000; 157:411–421. [PubMed: 10934146]
40. Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL. Cancer. 2000; 88:2606–2618. [PubMed: 10861440]
41. Hoffman MA, Ohh M, Yang H, Klco JM, Ivan M, Kaelin WG Jr. Hum. Mol. Genet. 2001; 10:1019–1027. [PubMed: 11331612]
42. Jiang BH, Semenza GL, Bauer C, Marti HH. Am. J. Physiol. 1996; 271:C1172–C1180. [PubMed: 8897823]

43. Warburg O. Science. 1956; 123:309–314. [PubMed: 13298683]

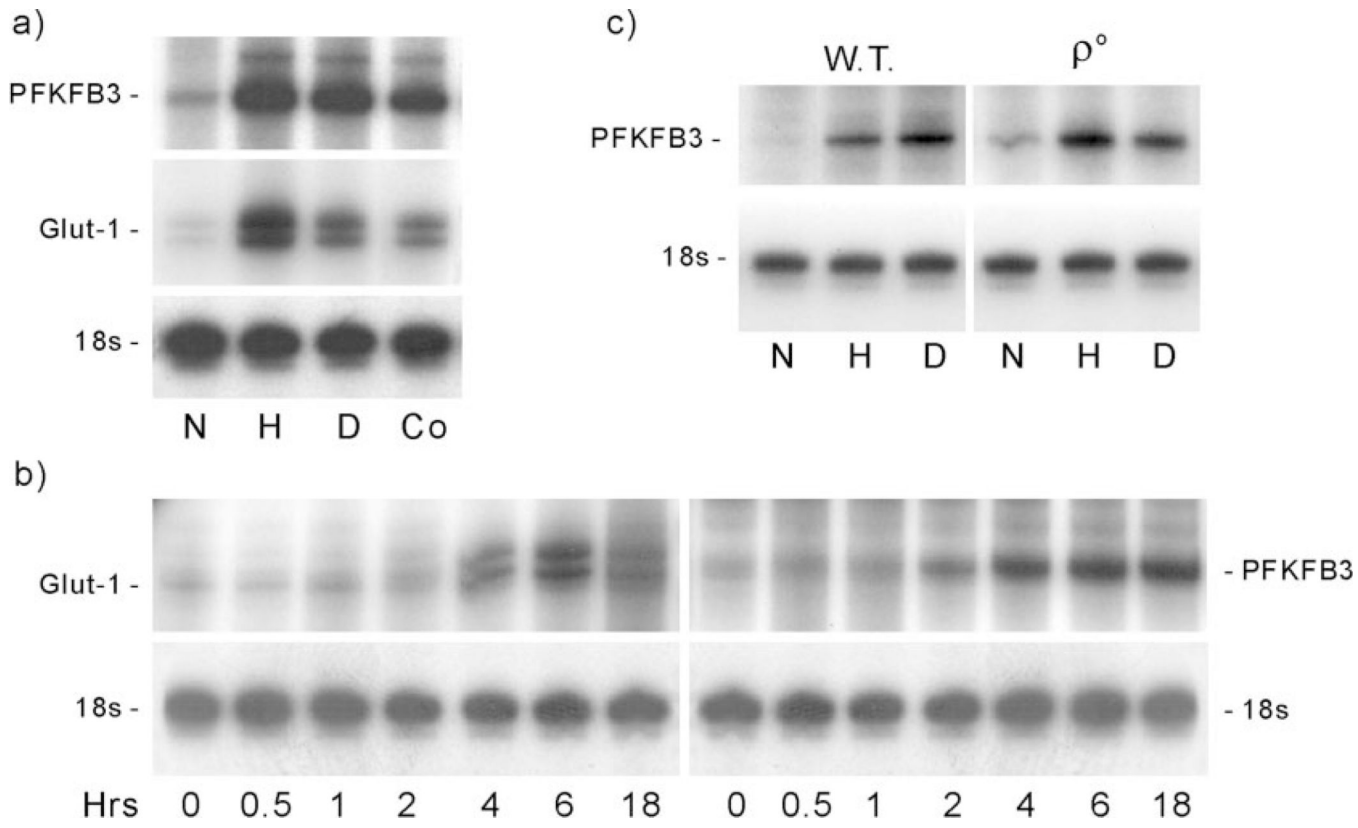

Fig. 1. Response of the *PFKFB3* gene to hypoxia, cobalt, and desferrioxamine

a, Hep-3B cells were maintained in normoxia (*N*), exposed to hypoxia (*H*), 0.5% O_2, or incubated with 100 μM cobalt chloride (*Co*) or 130 μM desferrioxamine (*D*) for 6 h. Total RNA was extracted and analyzed for PFKFB3 and Glut-1 mRNA expression using RNase protection assays. *b*, Hep-3B cells where treated with desferroxamine, and total RNA was isolated at various times (*Hrs*) following the treatment and analyzed for Glut-1 (*left*) or PFKGFB3 (*right*) mRNA. *c*, responsiveness of the *PFKFB3* gene to hypoxia (*H*) and desferrioxamine in wild-type (*W.T.*) and ρ° cells (25). In all figures, *18s* represents 18 S ribosomal RNA.

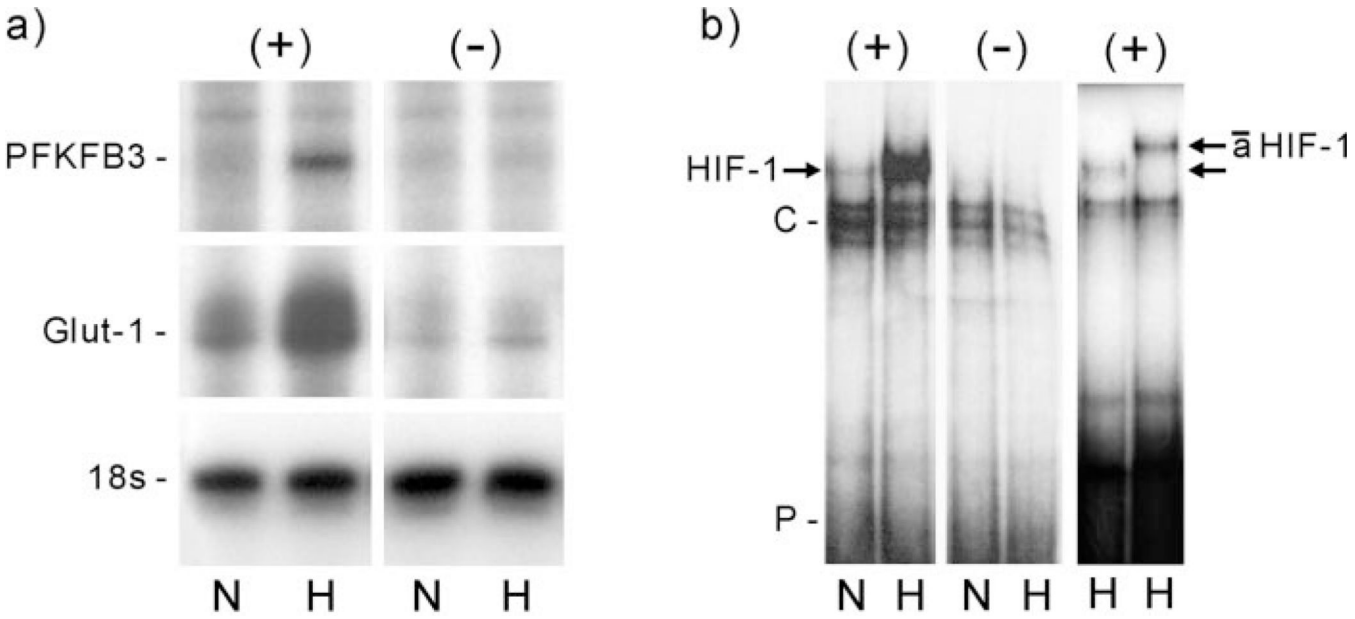

Fig. 2. Hypoxic response of the *PFKFB3* gene in HIF-1α negative cells

a, HIF-1α negative mouse fibroblasts (−) and their HIF-1α positive (+) controls (14, 34) where exposed to hypoxia for 6 h, and the total RNA was analyzed for PFKFB3 and Glut-1 mRNA by RNase protection assays. *b*, electrophoretic gel shift assay of nuclear extracts from HIF-1α (+) and HIF-1α (−) cells exposed to normoxia (*N*) or hypoxia (*H*) using a probe (*P*) that contains the erythropoietin HRE. *C* represents constitutive bands. The *rightmost panel* shows a supershift assay in hypoxic HIF-1α (+) cells utilizing anti-HIF-1α antibodies.

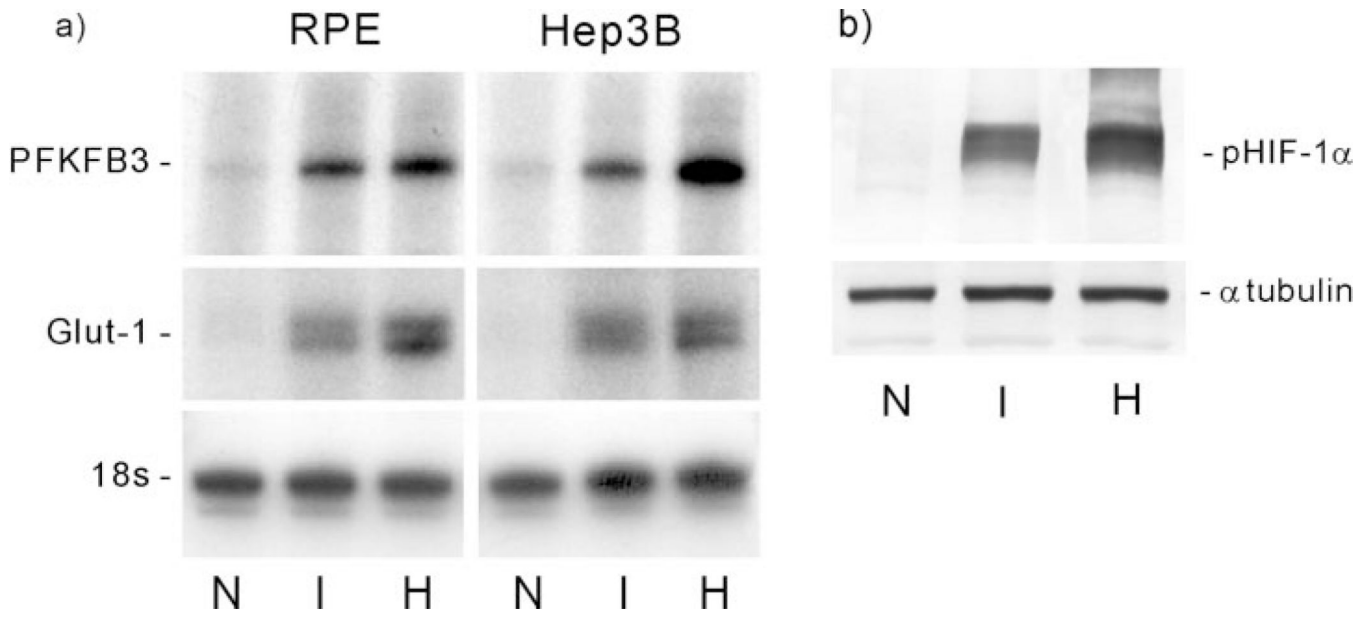

Fig. 3. Effect of dimethyloxalylglycine on PFKFB3 mRNA expression

a, confluent monolayers of hepatoma (*Hep3B*) or retinal pigment epithelial (*RPE*) cells where maintained in normoxia (*N*) or exposed to 1 mM dimethyloxalylglycine (*I*) or hypoxia (*H*) for 6 h. Total RNA was analyzed for PFKFB3 and Glut-1 expression by RNase protection assay. *b*, Western blot of Hep-3B cells treated as above and analyzed for HIF-1α expression utilizing anti-HIF-1α monoclonal antibody.

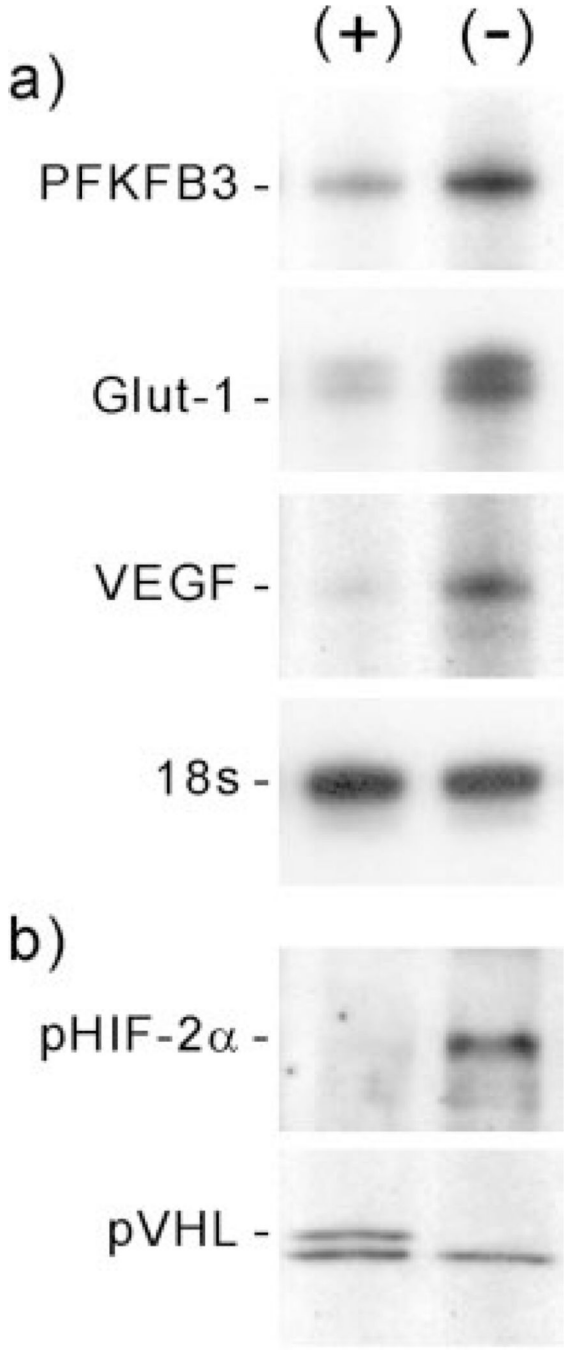

Fig. 4. Expression of the *PFKFB3* gene in pVHL-deficient cells

a, total RNA from normoxic VHL deficient (−) renal carcinoma 786-0 cells and their VHL positive (+) controls where analyzed for PFKFB3, Glut-1, and VEGF mRNA expression by RNase protection assays. *b*, whole cell extracts from VHL (+) and VHL (−) were analyzed by Western blots using anti-HIF-2α and anti-VHL antibodies.