Phosphatidylinositol 3-Kinase/Akt Signaling Is Neither Required for Hypoxic Stabilization of HIF-1α nor Sufficient for HIF-1-dependent Target Gene Transcription

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The serine/threonine kinase Akt/PKB and the oxygen-responsive transcription factor HIF-1 share the ability to induce such processes as angiogenesis, glucose uptake, and glycolysis. Akt activity and HIF-1 are both essential for development and implicated in tumor growth. Upon activation by products of phosphatidylinositol 3-kinase (PI3K), Akt phosphorylates downstream targets that stimulate growth and inhibit apoptosis. Previous reports suggest that Akt may achieve its effects on angiogenesis and glucose metabolism by stimulating HIF-1 activity. We report here that, whereas serum stimulation can induce a slight accumulation of HIF-1α protein in a PI3K/Akt pathway-dependent fashion, hypoxia induces much higher levels of HIF-1α protein and HIF-1 DNA binding activity independently of PI3K and mTOR activity. In addition, we find the effects of constitutively active Akt on HIF-1 activity are cell-type specific. High levels of Akt signaling can modestly increase HIF-1α protein, but this increase does not affect HIF-1 target gene expression. Therefore, the PI3K/Akt pathway is not necessary for hypoxic induction of HIF-1 subunits or activity, and constitutively active Akt is not itself sufficient to induce HIF-1 activity.

Oxygen (O₂) is critical for mammalian cells, which have evolved a variety of molecular mechanisms to sense and respond to both physiologic and pathophysiologic changes in O₂ (1). From the standpoint of cellular survival, lack of O₂ (hypoxia) requires a metabolic switch from oxidative to glycolytic metabolism coupled with corresponding changes in the vasculature that compensate for reduced ATP-generating efficiency. To accomplish this, cells respond to low O₂ by transactivating genes that increase glucose uptake (GLUT1), glycolysis (glycolytic enzymes), red blood cell production (erythropoietin), and vasodilation (inducible nitric-oxide synthase). However, these responses are short term survival strategies and do not address the fundamental problem of O₂ deficiency. To restore O₂ delivery to starved tissues, new blood vessel growth, known as angiogenesis or neovascularization, is required. Angiogenesis, the spraying and growth of new vessels from existing ones, is activated primarily by vascular endothelial growth factor, a potent and highly endothelial cell-specific mitogen, whose expression is induced by hypoxia (2).

The transcriptional response to hypoxia is regulated by the hypoxia-inducible factors (HIFs), heterodimeric transcription factors consisting of a regulated α subunit (HIF-1α or HIF-2α/EPAS1) and a constitutive β subunit (ARNT/HIF-1β or ARNT2), all of which are bHLH-PAS proteins. The bHLH-PAS superfamily contains many proteins involved in sensing and responding to changes in environmental conditions (3), and the HIF family exemplifies this role (4). The HIF-α subunits are constitutively transcribed and translated, but protein levels are controlled by ubiquitination and proteasomal degradation. HIF-α subunits are ubiquitinated in direct proportion to cellular O₂ concentrations, providing a molecular rheostat whereby levels of hypoxia-responsive genes are finely and expeditiously regulated by O₂. Stabilized HIF-α translocates to the nucleus where they dimerize with a β subunit, usually ARNT, and the heterodimer then binds to hypoxia response elements (HREs) (5) in the promoters and enhancers of target genes resulting in their transactivation. HIF-mediated gene expression is critical for both embryonic development (6–8) and tumor growth (9, 10).

Much is known mechanistically about the way HIF-α subunits are modified and destroyed. Newly translated HIF-α is hydroxylated at two proline residues in its oxygen-dependent degradation domain (ODD) (11) by HIF-prolyl hydroxylases (12–17). This modification allows HIF-α to be recognized by a ubiquitin E3 ligase complex containing pVHL, a tumor suppressor that recognizes hydroxylated HIF-α and allows it to be targeted to the 26 S proteasome (18). In this fashion, low O₂ leads to the accumulation of HIF-α subunits. The HIF-prolyl hydroxylases are among many proposed oxygen sensors, including the mitochondria (19, 20), but where precisely cellular oxygen sensing takes place and by what remains a contentious issue.

While the mechanism of HIF-α degradation is becoming clear, the signaling pathways that influence HIF-α stability and nuclear translocation and, subsequently, HIF-1 transcriptional activity remain somewhat confusing and controversial. Many published reports suggest that the regulation of HIF-1α

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‡ The abbreviations used are: HIF, hypoxia-inducible factor; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; bHLH-PAS, basic helix-loop-helix/Per-Arnt-Sim homology; HRE, hypoxia response element; miR, mutant HRE; wHRE, wild type HRE; E3, ubiquitin-protein isopeptide ligase; VHL, von Hippel-Lindau; pVHL, VHL tumor suppressor protein; myr, myristoylated; FBS, fetal bovine serum; tet, tetracycline; EMISA, electrophoretic mobility shift assay; PGK, phosphoglycerate kinase; β-gal, β-galactosidase; LY, LY294002; EF1α, elongation factor 1α; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase.
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stabilization and nuclear translocation and HIF-1 transcriptional activity are distinct events that are coordinately controlled by hypoxia (4), although evidence also exists that hypoxic signal transduction is not necessary for HIF activity when HIF-1α is overexpressed (21) or constitutively stable (16).

Particular scrutiny has been focused on the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Akt (also known as protein kinase B or PKB), a proto-oncogene, is a major downstream effector of growth factor signaling and has a wide array of progresig, proapoptotic effects when activated by growth factors through PI3K (for review, see Ref. 22). Most intriguingly Akt shares with HIF-1 the ability to induce vascular endothelial growth factor and angiogenesis (23, 24) and to increase cellular glucose uptake and glycolysis (25–27). Many nonhypoxic stimuli including the inflammatory mediators NO, tumor necrosis factor-α (28, 29), and thrombin (30); growth factors including insulin (31), insulin-like growth factor (24, 32), epidermal growth factor (33), hepatocyte growth factor/ scatter factor (34), platelet-derived growth factor, and transforming growth factor-β (30); and heregulin (35) have been reported to induce HIF activity in PI3K-dependent or -related ways. In certain cell types, positive stimulation of the PI3K/Akt pathway using constitutively active molecules can induce or enhance HIF activity. Conversely inhibition of the pathway using dominant negative peptides, small molecule inhibitors, or overexpression of PTEN, a phosphatase that reverses PI3K-catalyzed phosphorylation, can block HIF activity (24, 32, 33, 36–39), although this remains controversial (40).

Previous reports suggest that PI3K and Akt are central in the regulation of HIF activity. We set out to test this link using a variety of different cell types and assays and to test both the necessity and sufficiency of Akt or PI3K activity to lead to HIF-1α stability or HIF-1 activity. We report here that, although inhibitors of the PI3K/Akt pathway can block serum-induced accumulation of HIF-1α, they do not block hypoxic induction. In transient transfections, constitutively active (myristoylated) Akt (myrAkt) is able to increase HIF-dependent transcription in glioblastoma cells but not in hepatoma cells, suggesting a fundamental difference in the way these two cell lines respond to constitutive Akt activity. In addition, we show in a murine pro-B cell line (FL5.12) that even at elevated levels of Akt signaling, HIF-1α protein levels are only slightly affected, and target gene expression is not induced, indicating that Akt activity itself is not sufficient in these cells to stimulate HIF-1 target gene transcription.

**EXPERIMENTAL PROCEDURES**

**Tissue Culture Cells and Reagents**—The murine hepatoma lines 1c1c7 and B6Rc1 (obtained from ATCC), endothelial line MS-1 (generously supplied by M. Gee and W. Lee), rat fibroblast line Rat-1α (generously supplied by N. Hay), and human glioblastoma line U873 (obtained from the University of California San Francisco Neurosurgery Tissue Bank, Ref. 41) were propagated in Dulbecco’s modified Eagle’s medium (Cellgro), 10% FBS, 10 mM HEPES, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids (all from Invitrogen). The murine pro-B cell line FL5.12 (42, 43) was cultured in RPMI 1640 medium (Invitrogen), 10% FBS, 10 mM HEPES, 50 μM β-mercaptoethanol, 50 units/ml penicillin, 50 μg/ml streptomycin supplemented with Wehi-3B supernatant or recombinant murine interleukin-3 (R&D Systems). Creation of the tet-on myrAkt-expressing clones has been described previously (26). Tet-on clones and expressing cells were diluted with 1 μM doxycycline (Sigma) overnight prior to experiment.

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**Western Blots**—For all Western blot and EMSA analyses, 3.5–4 × 106 (1c1c7) or 2 × 106 (U873) cells were plated in 10-cm tissue culture dishes such that the density of the cells at the time of lysis was ~60–70% confluent. Cells were allowed to adhere overnight and were then starved for 3 h in 0% FBS and pretreated for 30 min with the indicated drug or Me6SO control. Cells were then stimulated with 5% FCS, 20% O2, or 10% FBS, 1.5% O2 for the indicated times. For hypoxic extracts, cells were manipulated inside a hypoxic chamber using phosphate-buffered saline that had been equilibrated to the hypoxic environment. Lysis was performed immediately at the bench in ambient air. Nuclear extracts were prepared using a modified Dignam protocol (44) with Buffer C further modified to contain 300 mM NaCl (45). Whole cell lysates were prepared using WCE buffer: 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 0.1% SDS, 20 mM β-glycerophosphate, 10 mM NaF, 250 μM NaVO4, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor (Roche Molecular Biochemicals). Extracts were electrophoresed, transferred, and immunoblotted according to standard protocols or the manufacturer’s instructions using 5% nonfat dry milk (Carnation) in Tris-buffered saline/Tween 20. Blots were incubated with primary antibody 1:1000, washed with TBS-T, and incubated for 30 min with HRP-conjugated secondary antibody (1:10 000). Membranes were scanned on a Storm PhosphorImager, and quantitations were performed using ImageQuant software.
serine 253 to alanines. Oligonucleotides were as follows (mutations underlined): T251A sense, 5'-ggccagacgagctcgaggtcgcggccgccgcatggaaacagggtccagggg; TSS251AAA sense, 5'-ggccagacgagctcgaggtcgcggccgccgcatggaaacagggtccagggg; TSS251AAA antisense, 5'-catggaaacagggtccagggg; TSS251AAA antisense, 5'-catggaaacagggtccagggg.

For transient transfections, cells were plated at 1 × 10^5 (1c1c7 and BpRc1) or 3.75 × 10^5 (U373)/well of six-well plates in 10% FBS medium minus penicillin/streptomycin. Cells were allowed to adhere overnight and were transfected the next morning using either LipofectAMINE2000 (1c1c7 and BpRc1) or LipofectAMINE Plus (U373) transfection reagents (Invitrogen) in 10% FBS minus penicillin/streptomycin, and each well was then washed, trypsinized, and replated on two 60-mm dishes in 10% FBS with penicillin/streptomycin. After overnight incubation, all dishes were switched to 0% FBS for 6 h and then placed in either a hypoxic chamber or a normal tissue culture incubator. Cells were lysed, all dishes were switched to 0% FBS for at least 30 min in 10% FBS minus penicillin/streptomycin, and each well was then washed, trypsinized, and replated on two 60-mm dishes in 10% FBS with penicillin/streptomycin. After overnight incubation, all dishes were switched to 0% FBS for 6 h and then placed in either a hypoxic chamber or a normal tissue culture incubator. Cells were lysed, and luciferase activity was analyzed after 20 h of treatment using a luciferase assay kit (Promega) according to the manufacturer's instructions.

Hepatocytes were transfected using 12.5 μl of LipofectAMINE2000, 15 ng of pCDNA/myrAkt or empty pCDNA3 vector, 2 μg of either wHRE or mHRE, and 0.5 μg of EF1α/β-gal. U373 cells were transfected with 15 μl of LipofectAMINE, 20 μl of plus reagent, 1 μg of wHRE or mHRE, 250 ng of pCDNA/myrAkt or empty vector, and 2 μg of EF1α/β-gal.

RESULTS

Effects of PI3K Pathway Inhibitors on HIF-1α and HIF-1—To determine whether various steps in the PI3K/Akt signal transduction pathway are required for hypoxic or nonhypoxic stabilization of HIF-1α, as has been suggested previously, we used pharmacological inhibitors of PI3K (LY294002 and wortmannin) and of mTOR (rapamycin), a downstream target of Akt previously implicated in the control of HIF-1α (35) (Fig. 1). We used the 1c1c7 mouse hepatocyte cell line in which we and others have extensively characterized HIF-1α-dependent hypoxic responses. In 1c1c7 cells, LY294002 was able to modestly inhibit hypoxia-induced HIF-1 DNA binding activity and HIF-1α protein accumulation in a dose-dependent manner (Fig. 2, A and B). Wortmannin is as effective as LY294002 in blocking serum-stimulated phosphorylation of Akt, and all three drugs effectively block serum-stimulated phosphorylation of p70 (Fig. 2C), which lies downstream of both Akt and mTOR and acts as a mediator of PI3K/Akt modulation of protein translation (for review, see Ref. 48). While rapamycin may modestly inhibit HIF-1α accumulation, possibly due to its general effects on protein translation, neither rapamycin nor wortmannin had any dose-dependent effect on HIF-1 DNA binding activity or HIF-1α levels, suggesting that the effects of LY294002 were PI3K-independent. It therefore seems likely that the effects of LY294002 on HIF-1α are due to its effects on kinases other than PI3K. Differential effects of LY and wortmannin on cells have been observed previously (49).

A previous report demonstrated a role for the PI3K/Akt pathway in the control of HIF-1α in U373 human glioblastoma cells, which are mutated for the tumor suppressor PTEN (24). We found that, in U373 cells, the PI3K pathway inhibitors did not block hypoxic induction of HIF-1α DNA binding activity (Fig. 3A), although LY may slightly attenuate it as in the 1c1c7 cells. A slight increase in cellular HIF-1α levels was observed upon serum stimulation similar to that observed previously (Fig. 3B) (24). The serum-induced accumulation of HIF-1α is fully inhibited by all three drugs at all concentrations tested, consistent with the notion that this effect is a result of stimulation of protein translation by Akt and thus lies downstream of both Akt and mTOR (35). In contrast, hypoxic induction resulted in levels of HIF-1α protein substantially higher than those seen in serum stimulation (Fig. 3B) and was insensitive to any of the three drugs.

We also tested the effects of the drugs on HIF-1 induction by CoCl2, a common hypoxia mimetic, in three different cell lines. The drugs were ineffective in inhibiting induction of HIF-1α DNA binding activity in 1c1c7 hepatocytes, MS-1 endothelial cells, and rat-1a fibroblasts by CoCl2 (Fig. 4).

Thus, although blocking PI3K and mTOR activity can block serum induction of HIF-1α, their activities are not necessary for hypoxic or CoCl2-induced accumulation of HIF-1α protein or HIF-1 DNA binding activity, suggesting that the PI3K/Akt pathway is not necessary for hypoxic signal transduction. In support of this, in U373 cells hypoxia did not have any PI3K-dependent effects on phosphorylation of Akt or p70. In fact, there appears to be significantly less phospho-p70 in the hypoxic U373 cells compared with the appropriate normoxic control, demonstrating that mTOR-dependent control of translation does not play a role in the hypoxic stabilization of HIF-1α.

Effects of PI3K Pathway Inhibitors on HIF-1 Target Gene Levels and Hypoxic Induction—Stabilization of HIF-1α is an important step in the cell response to hypoxia, but ultimately the effectiveness of the hypoxia response depends on transcription of HIF-1 target genes. Our observations indicated that the PI3K/Akt pathway had little effect on hypoxic induction of HIF-1α protein or HIF-1 DNA binding, but the possibility remained that PI3K/Akt signaling could affect HIF-1 target gene expression by blocking a step downstream of HIF-1α stabilization. Treatment with LY and rapamycin, but not wortmannin, resulted in moderately decreased levels of GLUT1 and PGK mRNA, both known to be transcriptionally regulated by HIF-1, under both normoxic and hypoxic conditions in 1c1c7 cells (Fig. 5A). However, since both normoxic and hypoxic mRNA levels were still inhibited by LY and rapamycin, the degree of hypoxic transcription of each gene was not attenuated by the drugs. In fact, the magnitude of hypoxic induction was in each case modestly higher in treated cells than in untreated, suggesting that hypoxia could overcome, to some degree, the inhibition of mRNA levels by LY and rapamycin. The fact that the two drugs inhibited transcription to a similar degree without
decreasing the magnitude of hypoxic induction strongly suggests that the effect, while reproducible, is not achieved via a block in HIF-1 activation. In U373 cells, LY and, to a lesser degree, rapamycin inhibited gross levels of HIF-1 target gene transcription but also reduced levels of /H9251 -tubulin mRNA, leading to an apparent increase of target gene transcription when quantitated and normalized to tubulin (Fig. 5B). Although the effects of wortmannin on gene expression were marginal, it remains effective at blocking Akt phosphorylation even after 24 h of incubation at 37 °C, thus wortmannin stability is not an issue for this experiment (data not shown). Blocking PI3K and mTOR clearly had effects on gene expression, but these effects did not appear to be HIF-1-specific or HIF-1-dependent. In fact, LY and rapamycin had greater effects on /H9251 -tubulin in the U373 cells.

**Fig. 2.** *PI3K pathway inhibition does not block hypoxic induction of HIF-1 in 1c1c7 hepatocytes.* Serum-starved 1c1c7 hepatocytes were pretreated in the indicated concentrations of PI3K pathway inhibitors or in Me2SO (DMSO) for 30 min before incubation at 20 or 1.5% O2 for 6 h. 5 or 30 µg of nuclear extracts were subjected to EMSA (A) or Western blot analysis (B), respectively. In the EMSA, 0.5 µl of α-ARNT monoclonal antibody was added to the indicated lanes to ablate or supershift the DNA binding complex. Quantitation of HIF-1 band intensity relative to the hypoxia/Me2SO signal is noted below the appropriate lanes. For Akt and p70 Western blots (C), cells were pretreated in 10 or 30 µM, 20 or 50 nM rapamycin, 100 or 200 nM wortmannin, or Me2SO and serum-stimulated (10% FBS) for 45 min. 30 µg of whole cell extracts per lane were subjected to Western blot analysis for phospho-Akt (p-Akt) (Ser-473), p70, and phospho-p70 (p-p70) (Thr-389). The phospho-specific Akt antibody was then stripped, and the blot was reprobed with nonphospho-specific Akt antibody. Rap, rapamycin; Wort, wortmannin.

**Fig. 3.** *PI3K pathway inhibition blocks serum but not hypoxia induction of HIF-1 in U373 glioblastoma cells.* Serum-starved U373 glioblastoma cells were pretreated in the indicated concentrations of PI3K pathway inhibitors or in Me2SO (DMSO) and incubated in either 20 or 1.5% O2 for 3 h. EMSA was performed on 5 µg of nuclear extract (A). α-ARNT monoclonal antibody was added to the indicated lanes to ablate or supershift the DNA binding complex. Quantitation of HIF-1 band intensity relative to the hypoxia/Me2SO signal is noted below the appropriate lanes. For Western blots (B), cells were pretreated in 10 or 30 µM LY, 20 or 50 nM rapamycin, 100 or 200 nM wortmannin, or Me2SO and incubated in either 0% FBS, 20% O2, 10% FBS, 20% O2, or 0% FBS, 1.5% O2 for 3 h. 30 µg of whole cell extract per lane were subjected to Western blot analysis for HIF-1α, phospho-Akt (p-Akt) (Ser-473) and phospho-p70 (p-p70) (Thr-389). The phospho-specific antibodies were then stripped, and blots were reprobed with the corresponding nonphospho-specific antibodies for each protein. Light and dark exposures of HIF-1α are shown to illustrate the difference in levels between serum and hypoxia induction and to show more clearly the lack of inhibition by the drugs. Rap, rapamycin; Wort, wortmannin.
cells than on HIF-1 targets. Based on these results, it appears that, under hypoxic conditions, PI3K pathway regulation of HIF-1 target gene expression is not mediated through alterations in HIF-1 levels or transcriptional activity.

Myristoylated Akt Enhances HIF-1-dependent Transcription in U373 but Not 1c1c7 Cells—Other reports have demonstrated that a variety of activating stimuli in the PI3K/Akt pathway can increase HRE-dependent transcription (24, 32, 33). In 1c1c7 cells, cotransfection of myrAkt with an HRE-driven luciferase reporter did cause an increase in overall normoxic and hypoxic luciferase activity over vector controls when normalized to protein concentration (Fig. 6A). However, this effect was not HIF-1-dependent as it was also observed with a mutant reporter construct (mHRE) to which HIF-1 cannot bind and which retains no hypoxia-inducible activity. The effect of myrAkt on the mutant HRE reporter was roughly equivalent in magnitude to its effect on the wild type HRE reporter. MyrAkt expression also induced, by roughly 50%, reporter activity from a wide array of non-HRE-containing promoters, including β-actin, Maloney sarcoma virus, and human EF1α, demonstrating that myrAkt has significant effects on general cellular transcription and translation (data not shown). Thus, when luciferase levels were properly normalized to account for transfection efficiency and general increases in transcription or translation, in this case to an EF1α-driven β-gal reporter, the induction by myrAkt vanished.

Similar experiments carried out in the U373 cell line (Fig. 6B) showed a different result. In these cells, myrAkt induced HRE-luciferase activity in a HIF-1-dependent fashion as evidenced by the absence of any increase in the activity of the mHRE construct when cotransfected with myrAkt. This HIF-1-dependent induction persists, although slightly lessened, when normalized to β-gal activity, showing that myrAkt can enhance HRE-dependent and HIF-1-specific transcriptional activity in the U373 cells. It is important to note that, in both the 1c1c7 and U373 cells, the increase in HRE activity caused by myrAkt is observed under both normoxic and hypoxic conditions. The magnitude of hypoxic induction remains unchanged, strongly suggesting that myrAkt is not amplifying hypoxic signal transduction per se but is increasing reporter activity downstream of HIF-1 stabilization and DNA binding.

The differing effects of myrAkt in the two different cell lines suggests two things. First it shows that any interaction between Akt and HIF-1 is not obligatory. Second it points to the potential effects of mutational background in predisposing certain cell lines or cell types to myrAkt modulation of HIF-1 activity (see “Discussion”).

ARNT Does Not Require Its Consensus Akt Phosphorylation Site for Activity—Akt is a protein kinase that phosphorylates many substrates containing Akt consensus phosphorylation target sites (loosely defined as RXRXX(S/T)) (22). Akt phosphorylation of downstream targets can have a wide range of reg-

**FIG. 4.** PI3K pathway inhibition does not block cobalt stimulation of HIF-1 DNA binding activity. Rat-1a (A), MS-1 (B), or 1c1c7 (C) cells were plated and allowed to recover overnight and were roughly 60% confluent at the time of treatment. Dishes were pretreated in 10 μM LY294002, 20 nM rapamycin, 100 nM wortmannin, or Me2SO (DMSO) for 30 min before being incubated in 100 μM CoCl2 for 6 h. 7.5 μg of nuclear extracts were run per lane, and α-ARNT antibody was added where indicated. Rap, rapamycin; Wort, wortmannin.
ulatory effects from changing the subcellular localization of the substrate to activating or inactivating the enzymatic or kinase activity of the substrate. Murine ARNT contains a consensus Akt phosphorylation site (RMRCGT) between amino acids 246 and 251 that is conserved in rat and human ARNT, offering the attractive hypothesis that Akt modulates HIF-1 activity through direct phosphorylation of the ARNT subunit. Point mutations of ARNT were thus constructed in which the putative target threonine or the threonine and two adjacent serines were changed to alanine. Using BpRc1 cells, an ARNT-deficient derivative of the 1c1c7 hepatocyte line (50, 51), we transiently transfected either wild type ARNT or one of the two ARNT mutants in the presence or absence of cotransfected myrAkt. We also transfected ARNT2, a closely related family member that does not have the Akt target site. Since the BpRc1 line has no endogenous ARNT, and thus no HIF-1 activity, we assessed the ability of the transfected cDNAs to restore hypoxia inducibility to an HRE-luciferase construct and the ability of myrAkt to modulate that restoration. The wild type and mutant ARNT proteins are each fully competent to restore HRE-dependent, hypoxia-inducible transcription (Fig. 6C). MyrAkt, while boosting transcriptional activity in each experiment, had the same effect irrespective of which ARNT construct was cotransfected and, in fact, increased HRE activity in the absence of ARNT, again showing the HIF-1 independence of this effect in the 1c1c7 line and its derivatives. While one other consensus Akt site does exist within murine ARNT, this site is not conserved in rat or human homologs, suggesting that it is not functionally important. The conserved Akt consensus site within ARNT is therefore not necessary for full HIF-1 transcriptional activity or for Akt-mediated effects on reporter expression.

**DISCUSSION**

It has been tempting to speculate, based on published reports, that the demonstrable interactions between HIF-1 and the PI3K/Akt/mTOR pathway represent a common mechanism...
whereby growth factor signaling or oncogenic transformation could lead to increased angiogenesis, glucose transport, and glycolysis. Constitutively active Akt and PI3K have remarkable effects on angiogenesis in chick chorioallantoic membrane assays (23) and on metabolism in mammalian cells (25–27). HIF-1 transactivates many genes that would be useful to a rapidly proliferating cell mass, including genes and processes known to be impacted by Akt, and is regulated post-translationally in a way that could easily be modulated by a kinase such as Akt. In addition, both HIF-1 and Akt are responsive to the environment of the cell, enacting cellular responses to changes in the cellular milieu. The fact that so many of the responses enacted by growth factor signaling through PI3K and Akt overlap with those regulated by HIF-1 makes HIF an attractive candidate as a downstream effector of Akt.

In fact, HIF-1α protein and/or HIF-1 DNA binding levels have been shown to be responsive to a variety of growth factors and oncogenes in certain cell types (for review, see Ref. 4), and these responses are often PI3K- and mTOR-dependent. Constitutively active PI3K and Akt, as well as loss of PTEN, appear to enhance HIF-1 activity (24, 32, 33). In addition, overexpression of wild type PTEN in U373 glioblastoma cells, normally mutated for this gene, can completely ablate hypoxic induction of HIF-1 (24). It has been shown that growth factors and oncogenes can induce HIF-1α, but we demonstrate here that this induction is of a much lower magnitude than hypoxia and is context- and cell type-dependent in marked contrast to hypoxia. Previous reports have relied on prostate cancer and glioblastoma lines, which are known to have high mutation frequencies in the PTEN gene and elsewhere in the PI3K pathway (52). The mutational backgrounds of these cells may predispose or sensitize them to additional perturbations in the PI3K pathway. As we have shown in hepatocytes and in glioblastoma cells, PI3K activity is not necessary for hypoxic accumulation of HIF-1α or activation of HIF-1, and in FL5.12 cells myrAkt does not affect HIF-1 target genes. A recent report from Semenza and colleagues (35) convincingly shows that, in breast cancer and 3T3 cells, heregulin and HER2 stimulation of HIF-1α occurs via an increase in the rate of protein synthesis not by blocking its degradation. This is an attractive model for all growth factor-induced increases in HIF-1 activity since it invokes a mechanism that does not involve VHL, HIF-prolyl hydroxylase, or hypoxia.

Many signaling pathways other than PI3K/Akt have been

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**Fig. 6.** MyrAkt can enhance HIF-1 activity in U373 but not 1c1c7 cells. 1c1c7 (A) or U373 (B) cells were transfected with wild type or mutant HRE reporters (wHRE or mHRE, respectively), a β-gal control plasmid (EF1α/β-gal), and either pCDNA3/myrAkt or empty vector. Lysates were assayed for protein concentration, luciferase activity, and β-gal activity. Luciferase values were normalized to either protein concentration (top panels) or β-gal activity (bottom panels) and are graphed as a function of normoxic wHRE activity in the absence of myrAkt. Hypoxic induction represents the -fold change between normoxia and hypoxia within each transfection. Each bar represents the mean of three separate transfections ± S.E. BpRc1 cells (C) were transfected with wHRE or mHRE, the indicated HIF-β subunit or vector, and myrAkt or vector. Values shown are normalized to protein concentration and graphed as a function of normoxic activity in ARNT/wHRE-transfected cells.
studied with respect to hypoxic signal transduction and HIF activity, and it has been proposed that HIF-1α stability, translocation, dimerization with ARNT, and transcriptional activity are separable (and perhaps separately regulated) events. MAPK/MEK/ERK (21, 39, 53–57) c-Jun NH₂-terminal kinase/p38 (34, 56), Ras (37, 58), and Rac1 (59) have all been implicated in aspects of HIF-1 regulation. In worms and flies, however, the loss of HIF-prolyl hydroxylase activity prevents HIF-1α stabilization and causes HIF-1 target genes to be up-regulated as well (12, 13). These results suggest that hypoxic signaling is not necessary for HIF-1 activity. In support of this, VHL-null tumor lines (14, 15) and embryonic stem cells² possess constitutively stable and transcriptionally active HIF-1. In addition, rendering HIF-1α constitutively stable (16) or overexpressing it under normoxic conditions are each sufficient to activate target genes, although the latter appears to be partially dependent on MAPK for full activity (21). Thus it appears that HIF-1α is at least partially competent to activate transcription of target genes in the absence of any additional hypoxic signaling. The activities of other pathways implicated in or activated by hypoxic signaling may be required for additional activation but do not appear to be necessary for basal function.

We show here that 1c1c7 hepatocyte and U373 glioblastoma cell lines, both of which have robust HIF responses, behave differently with respect to myrAkt. While myrAkt can induce modest HIF-dependent transcriptional responses in the U373 cells, it is unable to do so in the 1c1c7 line. In addition, FL5.12 cells, which show marked survival and metabolic phenotypes in response to 1c1c7 (26), demonstrate no consistent HIF-1α response and no HIF-1 target gene induction by increased myrAkt expression. This suggests both that myrAkt alone is not sufficient to induce HIF-1 and that the metabolic and antiapoptotic effects of myrAkt in these cells are not dependent on HIF-1.

² F. Mack, personal communication.

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**FIG. 7.** Elevated myrAkt can augment HIF-1α protein levels but not HIF-1 transactivation of target genes. **A**, after 6 h of normoxia or hypoxia, whole cell extracts were prepared and 75 μg (HIF-1α) or 20 μg (all others) were run on 8% SDS-polyacrylamide gels. Two independent clones of tet-myrAkt are shown. **B**, cells were incubated in either 20 or 1.5% O₂ for 16 h, and total RNA was extracted. 20 μg of RNA was run per lane and serially probed for PGK, GLUT1, and α-tubulin. **C**, cells were incubated in either 20 or 1.5% O₂, and 20-ml aliquots (~6 x 10⁶ cells) were removed for RNA preparation at the indicated times. 20 μg of total RNA was run on a Northern and probed for GLUT1, then stripped, and reprobed for tubulin. GLUT1 message levels were normalized to tubulin and expressed as a function of GLUT1/tubulin in the vector cells at t = 0 (graph). At the conclusion of the experiment, whole cell extract was made from the remaining cells, and 20 μg was run on 8% SDS-polyacrylamide gels and immunoblotted (D). The arrow indicates the myrAkt band. p-, phospho-.
Hypoxia and Akt have profound effects on cellular glucose metabolism, leading to increased glucose uptake and lactate secretion over time. Consequent changes in the culture medium can themselves affect gene expression and must be controlled for. In our experimental systems, the differences between glucose and lactate in the medium of normoxic and hypoxic 1c1c7 cells is less than 5% at 24 h (data not shown). In the myrAkt-expressing FL5.12 cells, there is a slightly more significant change in glucose and lactate (on the order of 10%) compared with vector controls at 24 h. However, we do not believe that these changes are sufficiently pronounced to alter the metabolism of the cells over the time periods studied as the medium is still replete with glucose after 24 h, and any accumulation of lactate is similar between Akt and vector control cells. In addition, we feel that in any experiments where these factors might affect the results, the appropriate controls (i.e. ± oxygen, ± hypoxia, and ±Akt) have been included to rule out such variables.

Hypoxia induces HIF-α levels in all cell lines or primary cell cultures tested (except those lacking pVHL), demonstrating the importance and primacy of hypoxic regulation of HIF. MyrAkt, in contrast, seems only to affect HIF in certain cell types. Whether this is a function of PTEN mutation or other dysregulation of the PI3K pathway or through inactivation of the constitutive up-regulation of PI3K signaling, either through onco-genic activation of the pathway or through inactivation of the PTEN tumor suppressor, can lead to increased HIF-α levels in certain tumor contexts. However, the relevance of this to non-tumor-related HIF function remains unclear. There may be important distinctions between physiological and pathophysiological regulation and effects of HIF-1, and further understanding of the role of the HIF family in both normal development and physiology and in tumor biology will depend on elucidation of these distinctions.

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Note Added in Proof—While this manuscript was under review, del Peso and colleagues reported similar findings using different cell lines and experimental approaches (Alvarado-Torajado, M. A., Aragonés, J., Vara, A., Landazuri, M. O., and del Peso, L. (January 28, 2002) J. Biol. Chem. 10.1074/jbc.M20017200).

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