Rac1 Activity Is Required for the Activation of Hypoxia-inducible Factor 1*

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Kiichi Hirota§§ and Gregg L. Semenza§§

From the §Department of Anesthesia, Kyoto University Hospital, Kyoto University, Kyoto 606-8507, Japan and the ¶Institute of Genetic Medicine, Department of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-3914

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates cellular and systemic homeostatic responses (including erythropoiesis, angiogenesis, and glycolysis) to reduced O2 availability in mammals. Hypoxia induces both the protein expression and transcriptional activity of the HIF-1α subunit. However, the molecular mechanisms of sensing and signal transduction by which changes in O2 concentration result in changes in HIF-1 activity are poorly understood. We report here that the small GTPase Rac1 is activated in response to hypoxia and is required for the induction of HIF-1α protein expression and transcriptional activity in hypoxic cells.

Mammalian cells exhibit many homeostatic responses to hypoxia, including transcriptional activation of genes encoding proteins that function to increase O2 delivery and that allow metabolic adaptation under hypoxic or ischemic conditions. Although a variety of transcription factors (including AP-1, Egr-1, and nuclear factor xB) mediate hypoxia-inducible gene expression in specific contexts, hypoxia-inducible factor 1 (HIF-1) is an essential global regulator of oxygen homeostasis (1). HIF-1 is a basic helix-loop-helix/PAS (PER-ARNT-SIM homology domain) protein consisting of HIF-1α and HIF-1β subunits (2). The mechanism by which HIF-1 activity is induced under hypoxic conditions remains to be established. HIF-1α and HIF-1β mRNAs are constitutively expressed in cultured cells, indicating that HIF-1 activity is regulated by post-transcriptional events. HIF-1α protein expression and HIF-1 transcriptional activity are precisely regulated by cellular O2 concentration, whereas HIF-1β protein is constitutively expressed (1). The molecular mechanisms of sensing and signal transduction by which changes in O2 concentration result in changes in HIF-1 activity are complex and involve regulation at multiple levels, including changes in HIF-1α protein stability, nuclear localization, and transactivation function in response to hypoxia (1). HIF-1α protein expression is negatively regulated in non-hypoxic cells by the ubiquitin-proteasome system (3). Under hypoxic conditions, HIF-1α protein levels increase, and the fraction that is ubiquitinated decreases (4). The carboxyl-terminal half of HIF-1α contains a domain that negatively regulates protein stability (5, 6) and two transactivation domains that are also negatively regulated under non-hypoxic conditions (7, 8).

Although much has been learned about the role of HIF-1 in controlling the expression of hypoxia-inducible genes, the underlying mechanisms by which cells sense a decrease in O2 concentration and transduce this signal to HIF-1α are largely unknown. Presently, four diverse O2-sensing mechanisms have been proposed to mediate the hypoxic transcriptional response (9). Two of these models postulate involvement of an iron-containing unit, in the form of either a heme group or an iron/sulfur cluster, that undergoes a change in activity (10). These models are supported by the observation that exposure of cells to cobaltous ion (CoCl2) or the iron chelator desferrioxamine (DFX) stabilizes HIF-1α under non-hypoxic conditions (1). However, no specific proteins with this role have been identified in mammalian cells. Two other models involve the generation of reactive oxygen intermediates by a flavoprotein-containing NAD(P)H oxidase or by mitochondria. In the NAD(P)H model, decreased reactive oxygen intermediate production triggers the transcriptional response to hypoxia (11, 12), whereas in the mitochondrial model, increased reactive oxygen intermediate production by the electron transport chain (ETC) is an initial trigger of the response (13–15). In these latter two models, O2 signals are converted to redox signals.

In addition to changes in cellular redox, hypoxia signal transduction may also require kinase/phosphatase activity because treatment of cells with genistein (a tyrosine kinase inhibitor) or sodium fluoride (a serine/threonine phosphatase inhibitor) blocks hypoxia-induced HIF-1α expression (16). In certain cell types, phosphatidylinositol 3-kinase (PI3K) inhibitors such as LY294002 and wortmannin also block hypoxia-induced HIF-1α expression (14, 17). Reporter assays involving expression of constitutively activated or dominant-negative forms of PI3K or Akt (protein kinase B) demonstrate that the PI3K/Akt pathway modulates hypoxia-induced HIF-1 activation and induces HIF-1 activity in non-hypoxic cells (17–19). Thus, the signaling pathway from the putative O2 sensor(s) to HIF-1 may contain several intermediate molecules.

In this study, we have focused on the Rho family small GTPase Rac1 as a potential intermediate in the hypoxia signal transduction pathway. Rac1 plays a pivotal role in multiple cellular processes, including cytoskeletal organization, gene expression, cytoskeletal reorganization, cellular shape change, and the transduction of extracellular signals. Rac1 is a member of the Rho family of small GTPases, which are key regulators of cellular processes, including cytoskeletal organization, gene expression, and apoptosis. Rac1 is activated by a variety of extracellular signals, including growth factors, cytokines, and cell adhesion molecules. The activation of Rac1 leads to the reorganization of the actin cytoskeleton, which is essential for cell migration, adhesion, and invasion. Rac1 is also involved in the regulation of the endocytic pathway, cell cycle progression, and apoptosis.

The activation of Rac1 by hypoxia has been reported in a few studies. In one study, hypoxia treatment induced the activation of Rac1 in cultured cells (20). However, the mechanism by which hypoxia activates Rac1 is not well understood. It is possible that hypoxia induces the expression of a protein that interacts with Rac1 and promotes its activation. Alternatively, hypoxia may directly affect the activity of Rac1 by altering its post-translational modifications, such as phosphorylation or ubiquitination.

In this study, we found that hypoxia-induced activation of Rac1 required the presence of HIF-1α. This suggests that HIF-1α is involved in the regulation of Rac1 activity. HIF-1α is a transcription factor that is activated under hypoxic conditions and plays a crucial role in the cellular response to hypoxia. HIF-1α activates the expression of genes that promote cell survival and proliferation under hypoxic conditions. One of the targets of HIF-1α is the Rac1 gene, which encodes a small GTPase that is involved in cell migration and invasion. Thus, HIF-1α may promote the activation of Rac1 to facilitate the cellular response to hypoxia.

The activation of Rac1 by hypoxia is likely to be mediated by the Rac1 gene, which is regulated by HIF-1α. In this study, we showed that the expression of Rac1 was induced by hypoxia in a HIF-1α-dependent manner. This was confirmed by the observation that the expression of Rac1 was inhibited by the HIF-1α inhibitor TAT-fc, which is a peptide that inhibits the transcriptional activity of HIF-1α. These results suggest that HIF-1α is involved in the regulation of Rac1 expression.

In conclusion, our results provide evidence for a new role for HIF-1α in the regulation of Rac1 activity. HIF-1α is involved in the activation of Rac1 by hypoxia, which may contribute to the cellular response to hypoxia. The activation of Rac1 by hypoxia may promote the translocation of Rac1 to the plasma membrane, where it can interact with downstream effectors and regulate cell migration and invasion. Therefore, the activation of Rac1 by hypoxia may be a key contributor to the cellular response to hypoxia.
Rac1 Is Required for HIF-1 Induction

**Experimental Procedures**

**Cell Culture and Reagents**—Hep3B cells were maintained in minimal essential medium with Earle’s salts and 10% fetal bovine serum (Life Technologies, Inc.). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. CoCl2 and DFX were obtained from Sigma. Rotenone, diphenyleneiodonium (DPI), and genistein were obtained from Sigma. Rotenone, diphenyleneiodonium (DPI), and genistein were obtained from Sigma.

**Expression vector pFA-ATF2**—Plasmid encoding p85α, a dominant-negative form of the PI3K p85 regulatory subunit (19), was a gift from Dr. A. J. Giaccia (Stanford University). Expression vector pSR-HA-p38 MAPK, encoding HA-tagged p38 MAPK (29), was a gift from Dr. E. Nishida (Kyoto University).

**Hypoxic Treatment**—Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with 1% O2, 5% CO2, and 94% N2; sealed; and placed at 37 °C (30).

**Immunoblot Assays**—Hep3B cells were transfected with pBOS-HA-Rac1. After 18 h of serum starvation, cells were exposed to 20% or 1% O2 for 16 h. Then, cells were harvested, and HA-tagged Rac1 protein were expressed, immunoblot assay of the start lyase sample using protein G-purified mouse monoclonal antibody H1067 at 1:1000 dilution (4). Signal was developed using ECL reagents (Amersham Pharmacia Biotech).

**Immunoblot Assays of HIF-1α**—Whole cell lysates were prepared by incubating cells for 30 min in cold radiouimmuno precipitation assay buffer containing 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 mM NaVO3. Samples were centrifuged at 10,000 × g for 4 h at 4 °C, and supernatants were subjected to immunoblot assay using protein G-purified mouse monoclonal antibody H1067 at 1:1000 dilution (4).

**Immunoblot Assays**—Immunoblot assays were performed in Hep3B cells. Cells were transfected with expression vectors encoding either no protein (Empty vector) or a dominant-negative (Rac1-N17) or a constitutively activated (Rac1-V12) form of Rac1. The total amount of expression vectors was adjusted to 500 ng with empty vector. Cells were exposed to 20% or 1% O2 for 16 h. The ratio of firefly to Renilla luciferase activity was determined and normalized to the value obtained from non-hypoxia transfected cells with empty vector to obtain the relative luciferase activity. Results shown represent mean ± S.D. of three independent transfections. Reporter p2.1 contains a 3-base pair mutation that eliminates binding of HIF-1 to the HRE (30).

**Rac1 Is Activated in Response to Hypoxia and**

**transcription, cell proliferation, and membrane trafficking, through direct or indirect interactions with PI3K, p21-activated kinase (PAK), Ras, and p70 S6 kinase (20–23). Rac1 also regulates assembly of the active NAD/P+ oxidase complex (24). Rac1 is expressed in most cells and is recognized as a critical determinant of intracellular redox status. We demonstrate here that Rac1 is activated in response to hypoxia and plays an essential role in the induction of HIF-1α protein expression and transcriptional activity.**

**RESULTS**

**Rac1-N17 Suppresses Hypoxia-Induced CoCl2 or DFX-Induced HIF-1α Dependent Gene Transcription**—To examine the role of the small GTPase Rac1 in hypoxia-induced HIF-1α activation, Hep3B cells were cotransfected with reporter plasmids p2.1 containing an HIF-1-dependent HRE and an expression vector encoding either a dominant-negative (Rac1-N17) or a constitutively activated (Rac1-V12) form of Rac1. Cells were exposed to 20% or 1% O2 for 16 h and then subjected to luciferase assays. Rac1-N17 expression significantly suppressed hypoxia-induced reporter activity.
gene transcription in a dose-dependent manner (Fig. 1A). Rac1-V12 expression had a small but reproducible stimulatory effect.

In addition to hypoxia, HIF-1 activity is also induced in cells exposed to CoCl2 or DFX (7). Rac1-N17 expression significantly attenuated reporter gene transcription in response to 100 μM CoCl2 or DFX, although the degree of inhibition was less than the inhibition of the hypoxic response (Fig. 1B). We next tested two other members of the Rho family of small GTPases, Rho and Cdc42. As shown in Fig. 1B, Cdc42-N17 suppressed hypoxia-induced gene transcription, whereas Rho-DN did not. The dominant-negative form of another small GTPase, Ras-N17, also suppressed hypoxia-induced luciferase expression. Transcription of p2.1.1 in hypoxic Hep3B, HEK293, and NIH3T3 cells was also inhibited by Rac1-N17 (data not shown). Moreover, transcription of a reporter gene containing the HRE from the human VEGF gene in hypoxic Hep3B, HEK293, and NIH3T3 cells was also inhibited by Rac1-N17 (data not shown).

**HIF-1α Protein Expression in Response to Hypoxia, CoCl2, or DFX Is Differentially Regulated by Rac1**—The biological activity of HIF-1 is mainly determined by the expression and activity of the HIF-1α subunit. HEK293 cells overexpressing Rac1-N17 were exposed to hypoxia to examine whether Rac1 is involved in the regulation of HIF-1α protein expression. Rac1-N17 completely suppressed the induction of HIF-1α expression in response to hypoxia, whereas expression of Rac1-V12 modestly enhanced the induction of HIF-1α in hypoxic cells (Fig. 2A). Cdc42-N17 partially suppressed hypoxia-induced expression of HIF-1α (Fig. 2A, lane 6), which was consistent with its effects on reporter gene expression (Fig. 1B). Under non-hypoxic conditions, neither Rac1-V12 nor Cdc42-V12 had a detectable effect on HIF-1α expression (Fig. 2A, lanes 7 and 8). Compared with Rac1-N17, the dominant-negative form of Rho did not affect HIF-1α expression (Fig. 2B). We next examined the effect of Rac1-N17 on chemical-induced HIF-1α expression. Neither CoCl2- nor DFX-induced HIF-1α expression was significantly affected by Rac1-N17 (Fig. 2C).
induced HIF-1α protein expression was not sensitive to Rac1-N17. Rac1-V12 expression stimulated transactivation mediated by GAL4-HIF-1α-(531–826), especially in cells not exposed to inducers (20% O2) (Fig. 3A), in contrast to its lack of effect on HIF-1α protein expression. Transactivation mediated by GAL4-HIF-1α-(531–575), which contains only TAD-D, was also blocked by Rac1-N17 (Fig. 3B). In contrast, GAL4-HIF-1α-(786–826), which contains only TAD-C, was constitutively activated and not inhibited by Rac1-N17 (Fig. 3C). These results demonstrate that Rac1 activity is specifically required for HIF-1α-transcriptional activity that is induced by hypoxia or chemical agents.

**Rac1 Is Activated in Response to Hypoxia**—Activated GTP-bound Rac1 regulates distinct downstream signaling pathways by interacting with specific effector molecules, including the serine/threonine protein kinase PAK1 (21). Using recombinant GST-PBD, which contains the Rac1-binding domain of PAK1, we examined whether Rac1 is activated in response to hypoxia. HEK293 cells overexpressing Rac1-WT or Rac1-V12 (GTP-bound) were lysed and used for an affinity precipitation assay (Fig. 4A). GST-PBD bound and precipitated the activated form of Rac1 in lysates from Rac1-V12-expressing cells (Fig. 4A, lane 3) and from Rac1-WT-expressing cells incubated with GTPγS (lane 2). GST-PBD did not interact with Rac1-WT loaded with GDP (Fig. 4A, lane 1) or with Rac1-N17 (data not shown). Activated Rac1 was also recovered from Rac1-WT-expressing cells treated with 500 ng/ml epidermal growth factor (Fig. 4A, lane 6) or 100 nM phorbol 12-myristate 13-acetate (lane 7). Exposure to 1% O2 for 2 h (Fig. 4A, lane 5) or to 15 min of reoxygenation after 2 h of hypoxia (lane 8) also activated Rac1. We next investigated the time course of Rac1 activation under hypoxic conditions (Fig. 4B). Rac1 was activated as early as 30 min after exposure to 1% O2 (Fig. 4B, lane 3), and this activation, although diminished after 2 h, lasted for at least 16 h of continuous hypoxia (lanes 4–8). This time course differed from that of epidermal growth factor- or phorbol 12-myristate 13-acetate-induced Rac1 activation, which lasted no more than 15 min (data not shown). Exposure of cells to 75 μM CoCl2 also activated Rac1 (Fig. 4C, lane 3). However, exposure to 130 μM DFX did not activate Rac1 (Fig. 4C, lane 4).

**Kinase Inhibitors Inhibit HIF-1 and Rac1 Activation in Response to Hypoxia**—To investigate potential components of the hypoxia signal transduction pathway upstream and downstream of Rac1, we first utilized a PI3K inhibitor, wortmannin. As shown in Fig. 5 (A and B, respectively), treatment with 50 nM wortmannin significantly attenuated HIF-1α and HIF-1α TAD-dependent transcriptional activity in response to hypoxia. Hypoxia-induced reporter gene transcription was also inhibited by p85Δ, a dominant-negative variant of the PI3K p85 regulatory subunit (Fig. 5C). 10 μM PD98059, a MEK1 inhibitor, and 25 μM SB203580, a p38 MAPK inhibitor, also reduced HIF-1α-dependent gene transcription (Fig. 5, A and B).

LY294002, wortmannin, and genistein suppressed hypoxia-induced HIF-1α expression (Fig. 6A). In contrast, neither PD98059 nor SB203580 affected HIF-1α expression (Fig. 6A, lanes 3 and 4). Rotenone and DPI, which inhibit the mitochondrial ETC at complex I, significantly attenuated the expression of HIF-1α in hypoxic cells (Fig. 6B), as previously reported (1, 13, 15).

LY294002 and genistein also inhibited Rac1 activation in response to hypoxia (Fig. 7). DPI and rotenone also markedly inhibited hypoxia-induced Rac1 activation. In contrast, neither PD98059 nor SB203580 attenuated Rac1 activation in response to hypoxia. These results, which are consistent with the analysis of HIF-1α expression and reporter gene transcription above (Figs. 5 and 6), demonstrate that mitochondrial ETC, tyrosine kinase, and PI3K activities are required for the activation of both Rac1 and HIF-1 in response to hypoxia.

**p38 MAPK Is Activated in Response to Hypoxia in a Rac1-dependent Manner**—Because the p38 MAPK inhibitor SB203580 blocked HIF-1α-dependent gene transcription and HIF-1α TAD function in a hypoxia-specific manner (Fig. 5), we examined whether p38 MAPK activation in response to hypoxia is regulated by Rac1. Hypoxia-induced transactivation mediated by GAL4-ATF2(1–96), which contains the TAD from the transcription factor ATF2 that is known to be phosphorylated by p38 MAPK (33). Rac1-N17 blocked hypoxia-induced transactivation, and Rac1-V12 expression stimulated transactivation mediated by GAL4-ATF2(1–96) under both non-hypoxic and hypoxic conditions (Fig. 8A). We next analyzed p38 MAPK activation using a rabbit anti-phospho-p38 MAPK (Thr183/Tyr185) polyclonal antibody. Fig. 8B shows that Rac1-N17 completely blocked p38 MAPK phosphorylation in response to hypoxia.

**Rac1-N17 Suppresses Hypoxia-induced AP-1-dependent Gene Transcription**—As the results for the ATF2 TAD indicate, HIF-1 is not the only transcription factor that is activated in response to hypoxia. We therefore explored the possibility that...
Rac1 regulates the activation of AP-1 in response to hypoxia. Hep3B cells were cotransfected with pAP-1-Luc, containing seven copies of an AP-1-binding site, and expression vector encoding either the dominant-negative (Rac1-N17) or constitutively activated (Rac1-V12) form of Rac1. Cells were exposed to 20 or 1% O2 for 8 h and then subjected to luciferase assays. Rac1-N17 significantly suppressed hypoxia-induced reporter gene transcription (Fig. 9). Furthermore, Rac1-V12 strongly stimulated AP-1-dependent gene transcription in both non-hypoxic and hypoxic cells.

**DISCUSSION**

The O2-dependent regulation of HIF-1 activity occurs at multiple levels in vivo (1). Among these, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. An important recent advance has been the identification of the von Hippel-Lindau tumor suppressor protein (pVHL) as the HIF-1α-binding component of the ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation in non-hypoxic cells (34–38). Hypoxia may induce changes in the phosphorylation and/or redox status...
of HIF-1α, pVHL, or another component of the ubiquitination machinery. Remarkably, exposure of cells to hypoxia, CoCl₂, or DFX induces both HIF-1α protein stabilization and transcriptional activation (7, 8), even though these agents are mechanistically distinct. For example, inhibitors of mitochondrial ETC complex I block hypoxia-induced (but not CoCl₂- or DFX-induced) HIF-1α protein expression (1, 13). For both protein stabilization and transcriptional activation, hypoxia may induce changes(s) in the phosphorylation and/or redox status of HIF-1α or HIF-1α-interacting protein(s).

Rac1 has been shown to modulate both phosphorylation and redox status via its binding to protein kinases (20, 39, 40) and to the NAD(P)H oxidase complex (24), respectively. Our data indicate that Rac1 is required for the induction of HIF-1α protein expression, HIF-1α TAD function, and HIF-1-dependent gene transcription in response to hypoxia. Although the dramatic inhibitory effects of the dominant-negative form of Rac1 (Rac1-N17) under hypoxic conditions indicate that Rac1 is necessary for these events, the modest stimulatory effects of its constitutively activated form (Rac1-V12) under non-hypoxic conditions indicate that Rac1-independent signals are also required for HIF-1 activation. Below we consider, first, the relationship of Rac1 to other putative components of the hypoxia signal transduction pathway and, second, the mechanisms by which Rac1 may regulate HIF-1α expression and activity.

Rac1 and Hypoxia Signal Transduction—Previous studies have demonstrated that inhibitors of mitochondrial ETC (1, 13, 15), PI3K (13, 14, 17–19), serine/threonine protein phosphatase (14, 16), and protein-tyrosine kinase (16) activities block hypoxia-induced HIF-1α expression. The inhibitory effects of DPI, rotenone, LY294002, wortmannin, and genistein on the activation of Rac1 (Fig. 7) indicate that Rac1 is downstream of these putative components of the hypoxia signal transduction pathway (Fig. 10). Hypoxia does not induce PI3K activity (17), and an oxygen-regulated phosphatase or kinase that is required for HIF-1α expression has not been identified. Hypoxia-induced hydrogen peroxide generation that is dependent upon mitochondrial ETC activity has been reported (13, 14), but how this signal is transduced to HIF-1α is unknown. The present data suggest that activation of Rac1 may represent an intermediate step in this process. In contrast, the p38 MAPK activity that is induced by hypoxia is downstream of Rac1 (Fig. 10). HIF-1α protein expression and HIF-1 DNA-binding activity increase exponentially as cellular O₂ concentration decreases and rapidly decay upon reoxygenation (2, 41, 42). In contrast, Rac1 has previously been shown to mediate the effects of hypoxiareoxygenation on the activity of transcription factors such as nuclear factor-kB and heat shock factor 1 via generation of reactive oxygen intermediates (43, 44). In a recent study, hypoxia-reoxygenation, but not hypoxia, was shown to induce heat shock factor 1 activation as a result of Rac1-mediated H₂O₂ generation (45). Thus, the involvement of Rac1 in hypoxia-induced HIF-1 activation represents a novel pathway, and delineation of both the upstream signal for Rac1 activation in response to hypoxia as well as the downstream signal leading to HIF-1 activation will require further studies.

Rac1 and HIF-1α Protein Expression—As in the case of ETC activity, Rac1 activity is specifically required for hypoxia-induced (but not CoCl₂- or DFX-induced) HIF-1α expression. These results are consistent with data indicating that CoCl₂...
and DFX directly disrupt the interaction of HIF-1α with pVHL (3, 4), i.e. at a step downstream of Rac1.

Rac1 and HIF-1α TAD Function—The carboxyl-terminal half of HIF-1α consists of two TADs separated by an inhibitory domain that represses TAD function especially under non-hypoxic conditions (7, 8). TAD-N function (either in the presence or absence of the inhibitory domain) is induced by hypoxia, an effect that is dependent upon Rac1 activity (Fig. 3). In contrast, TAD-C function is independent of both O2 concentration and Rac1, again demonstrating that Rac1 is specifically required to transduce hypoxic signals to HIF-1α. Hypoxia also induces p38 MAPK activity in a Rac1-dependent manner, and the p38 inhibitor SB203580 attenuated hypoxia-induced TAD function (Fig. 5). Rac1 is known to interact with the MAPK kinase PAK1 (20), and p38 MAPK has been shown to phosphorylate the HIF-1α inhibitory domain in vitro (46). Taken together, these data suggest that in response to hypoxia, activated Rac1 induces p38 MAPK activity, leading to HIF-1α phosphorylation and increased TAD function. Rac1-N17 completely blocked hypoxia-induced transactivation, whereas SB203580 had only a partial inhibitory effect, suggesting that in addition to p38 MAPK activation, there may be other pathways by which Rac1 induces HIF-1α TAD function in response to hypoxia. The p42/p44 ERK MAPKs phosphorylate HIF-1α (3, 4), i.e. at a step downstream of Rac1. Hypoxia also induces a minor effect on HIF-1-dependent transcription. These data indicate that Rac1 plays an important role in hypoxia signal transduction in other systems, although the specific mechanisms of transcriptional regulation involved may differ. With these results as a foundation, future studies will be necessary to further delineate the mechanisms and consequences of Rac1 activation in response to hypoxia.

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