Activation of Hypoxia-inducible Transcription Factor Depends Primarily upon Redox-sensitive Stabilization of Its α Subunit*

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Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor that is critical for hypoxic induction of a number of physiologically important genes. We present evidence that regulation of HIF-1 activity is primarily determined by the stability of the HIF-1α protein. Both HIF-1α and HIF-1β mRNAs were constitutively expressed in HeLa and Hep3B cells with no significant induction by hypoxia. However, the HIF-1α protein was barely detectable in normoxic cells, even when HIF-1α was overexpressed, but was highly induced in hypoxic cells, whereas HIF-1β protein levels remained constant, regardless of pO₂. Hypoxia-induced HIF-1 binding as well as the HIF-1α protein were rapidly and drastically decreased in vivo following an abrupt increase to normal oxygen tension. Moreover, short pre-exposure of cells to hydrogen peroxide selectively prevented hypoxia-induced HIF-1 binding via blocking accumulation of HIF-1α protein, whereas treatment of hypoxic cell extracts with H₂O₂ had no effect on HIF-1 binding. These observations suggest that an intact redox-dependent signaling pathway is required for destabilization of the HIF-1α protein. In hypoxic cell extracts, HIF-1 DNA binding was reversibly abolished by sulfhydryl oxidation. Furthermore, the addition of reduced thioredoxin to cell extracts enhanced HIF-1 DNA binding. Consistent with these results, overexpression of thioredoxin and Ref-1 significantly potentiated hypoxia-induced expression of a reporter construct containing the wild-type HIF-1 binding site. These experiments indicate that activation of HIF-1 involves redox-dependent stabilization of HIF-1α protein.

Hypoxia-inducible factor 1 (HIF-1)† was identified by Semenza, Wang, and co-workers (1–5) as a transcription factor in hypoxic cells that binds specifically to a 3′ enhancer of the gene encoding erythropoietin and to promoters/enhancers in other genes important in adaptation to hypoxia, such as those encoding tyrosine hydroxylase (6), vascular endothelial growth factor (7), glycolytic enzymes (8–10), and glucose transporters (11). HIF-1 activity can be induced by hypoxia in a wide variety of cells, as demonstrated by specific binding to oligonucleotides containing a HIF-1 response element (3) and by transactivation of reporter genes (12).

HIF-1 is composed of a 120-kDa α subunit and a 91–94-kDa β subunit (4). When activated, the HIF-1 heterodimer recognizes an 8-base pair DNA sequence 5′-TACGTGCT-3′ in the erythropoietin enhancer, interacting with both DNA strands in the major groove (13). Recent cloning of HIF-1α and HIF-1β genes (5) showed that they are members of the basic helix-loop-helix-PAS family of transcription factors. HIF-1α is a novel protein, whereas HIF-1β is the previously cloned and characterized aryl hydrocarbon receptor nuclear translocator (ARNT) (14).

HIF-1 DNA binding, along with the expression of HIF-1-responsive genes, can be induced by cobaltous ion and desferrioxamine with kinetics similar to that of hypoxia induction (3, 13). HIF-1 activation, irrespective of stimulus, is blocked by pretreatment of cells with cycloheximide (2, 3), an inhibitor of protein synthesis as well as by 2-aminopurine (13), a protein kinase inhibitor. Treatment of hypoxic cell extracts with alkaline phosphatase abolishes DNA binding (13). Thus phosphorylation may be required for binding. More recently, Wang et al. (5) reported that both HIF-1α mRNA and HIF-1β mRNA were barely detectable in normoxic cells but were highly induced following exposure to hypoxia, with rapid decay upon return to normoxia. Their results suggested that HIF-1 is regulated, at least in part, at the level of transcription and/or mRNA stability. However, these results are difficult to reconcile with the fact that both HIF-1α (15) and HIF-1β (ARNT) (14) genes were cloned independently from nonhypoxic cDNA libraries and that transcripts of both genes were detected at high levels. This concern prompted us to investigate the mechanism by which HIF-1 is regulated by first examining mRNA and protein expression of both α and β subunits. We found that levels of HIF-1α mRNA and HIF-1β mRNA were unaffected by changes in oxygen tension. In contrast, in vivo HIF-1 DNA binding activity induced by hypoxia closely paralleled the abundance of HIF-1α protein and that both HIF-1 activity and HIF-1α protein decreased markedly, not only by subsequent exposure of cells to oxygen, but also by pre-exposure of cells to hydrogen peroxide. The latter result led to our investigating the role of redox chemistry in HIF-1 activation.

EXPERIMENTAL PROCEDURES

Human HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 5% heat-inactivated fetal bovine serum, Hep3B cells in α-modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, and 293 cells in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum. Cells were routinely cultured in 95% air and 5% CO₂ at 37 °C and were made hypoxic by placing them in a gas-controlled chamber (Espec) maintained at 1% oxygen, 94% N₂, and 5% CO₂. In some experiments, cells were pretreated with 1 mM (or

*This work was supported by National Institutes of Health Grant RO1-DK41234 (to H. F. B) and grants from the National Institutes of Health and Dana Farber Cancer Institute/Sandoz Drug Development Program (to D. M. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Recipient of the National Research Service Award DK09365-01 from the National Institutes of Health.

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‡§Modified Eagle’s medium with 5% heat-inactivated fetal bovine serum, Hep3B cells in α-modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, and 293 cells in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum. Cells were routinely cultured in 95% air and 5% CO₂ at 37 °C and were made hypoxic by placing them in a gas-controlled chamber (Espec) maintained at 1% oxygen, 94% N₂, and 5% CO₂. In some experiments, cells were pretreated with 1 mM (or
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Plasmid Constructions—pBluescriptSK/HIF-1a3.2TT containing HIF-1α cDNA (kindly provided by G. L. Senzena) was modified by digestion with AvaI, filling in with Klenow fragment, and digestion again with XhoI. The blunt-ended AvaI-XhoI fragment was inserted into HindIII and XhoI sites of pBluescriptII SK+ to create pBS-HIF-1α. The resulting plasmid was further digested with HpaI and NsiI, blunt-ended as above, and religated to create pBS-HIF-1α/H through deletion of the 3' untranslated region. pBS-HIF-1α was also digested with Sphi and XhoI, blunt-ended as above, and religated to create pBS-HIF-1α/SX. Each plasmid contained appropriate stop codons for HIF-1α, Oct-1, or 1:5000 dilution of polyclonal anti-ARNT antibody (20), OZ15 (15) or 1:5000 dilution of polyclonal anti-ARNT antibody (20), followed by incubation into KpnI and XhoI sites of pcDNA3 (Invitrogen). For HIF-1α riboprobe, a 604-base pair EcoRI-EcoRI fragment was internally digested from pBS-HIF-1α/SX, resulting in pT3-HIF-1α. The human erythropoietin 3' enhancer region (3453–3500) containing a functional HIF-1 binding site was polymerase chain reaction-amplified and cloned into KpnI and SpeI sites of pBluescript vector (Promega Corp.). The resultant plasmid, pEpoOE-luc, carries a luciferase reporter gene driven by this 3' enhancer and SV40 promoter. To make pEpoEm1-luc, the HIF-1 site (5'-TACGTGCT-3') was mutated to 5'-TAAAAGCT-3' using the Altered Sites II (Promega Corp.) to generate a 623-nucleotide riboprobe, which protects 370 and 253 nucleotides of HIF-1α mRNA levels, cells were incubated under normoxic and hypoxic conditions. The possibility that longer hypoxic incubation might increase the level of HIF-1α stability was examined by incubating cells for 0, 1, or 2 days under normoxic or hypoxic conditions. As shown in Fig. 1, the level of HIF-1α protein was increased under hypoxic conditions, while it was not changed under normoxic conditions. Moreover, RNA obtained from Hep3B cells gave the same result (data not shown).

Preparation of Whole-cell Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—Cells were plated on ice, rinsed with phosphate-buffered saline, and harvested. Whole-cell extracts were prepared essentially as described previously (17). Briefly, cell pellets were quickly frozen in liquid nitrogen for 5 min and then thawed on ice for 5–10 min with cell lysis buffer (17). Cells were lysed at 4 °C by multiple (20) passages through a 26-gauge needle, followed by centrifugation at 12,000 × g for 15 min at 4 °C. The supernatants were stored at −80 °C. Protein concentrations were determined by using BCA protein assay reagent (Pierce). A typical DNA binding reaction was carried out by mixing 10 µg of cell extracts with DNA binding buffer (17) containing the presence of 150 ng of poly(dI-dC) and 1.75 pmol of M18 (mutant HIF-1 binding oligonucleotide) (2) for 5 min, followed by incubation for 10 min at room temperature with 17.5 fmol of 32P-labeled oligonucleotide containing the wild-type (W18) HIF-1 binding site (2), doublet bands (designated as HIF-1 binding) were detected in hypoxic extracts (Fig. 1, lane 2) consistent with previous reports (2, 3). In agreement with the evidence that HIF-1 is an αβ heterodimer, HIF-1 binding was supershifted with two different anti-HIF-1α monoclonal antibodies (lanes 5 and 6) and with a mixture of the two (lane 3), as well as with a polyclonal anti-HIF-1α antibody (lane 9).

To investigate the mechanism underlying regulation of HIF-1 activity, we first determined levels of HIF-1α and HIF-1β transcripts by ribonuclease protection analysis of HeLa cells maintained at 21% O2 (Fig. 2A, lane 1) or those incubated for 4 h under 1% O2 (lane 2). Contrary to the initial report on the cloning of the HIF-1 subunits (5), we found that both HIF-1α (Fig. 2A) and HIF-1β transcripts (data not shown) were readily detectable under normoxic conditions and were not significantly increased by hypoxia treatment. To exclude the possibility that longer hypoxic incubation might increase HIF-1α and HIF-1β mRNA levels, cells were incubated under 1% O2 for 16 h. Again, no significant induction was observed. Moreover, RNA obtained from Hep3B cells gave the same result (data not shown).

In contrast, hypoxia had a marked effect at the level of HIF-1α protein expression. When HeLa cells were incubated for 4 h in 1% O2, the induced induction in HIF-1α DNA binding activity (Fig. 2B, lane 2) was accompanied by a striking increase in HIF-1α protein from barely detectable (Fig. 2C, top panel, lane 1) to abundant expression (lane 2), whereas HIF-1β protein was constitutively expressed at a high level with no significant change in abundance following exposure to hypoxia (lower panel). One mechanism for the accumulation of HIF-1α protein is that at low oxygen tension, the subunit is stabilized.
FIG. 2. HIF-1 is sensitive to oxygen in vivo but stable in vitro. After a 4-h hypoxia treatment, HeLa cells were placed in a 21% oxygen incubator for 0, 5, 10, 30, and 60 min, respectively (lanes 2–6). A, total RNA was isolated to quantify HIF-1α mRNA by RNase protection analysis. Undigested riboprobes (undig pb) and protected HIF-1α fragments (623 nucleotides from incomplete digestion and 370 and 253 nucleotides from complete digestion) are marked. Lane 0 shows yeast tRNA mixed with the riboprobe. Cell extracts were prepared and analyzed by EMSA to examine HIF-1 binding (B) and Western blot to determine abundance of HIF-1α and HIF-1β (C). D, hypoxic extracts were incubated with [32P]W18 oligonucleotide at room temperature for 1, 5, 10, 20, and 40 min and then loaded onto an EMSA gel.

but is rapidly degraded when oxygen is replete. To address this question, cells were incubated under 1% O2 for 4 h, to produce maximal HIF-1 activation, and were then placed in a 21% O2 chamber for 5, 10, 30, or 60 min. As shown in Fig. 2B, there was a precipitous drop in HIF-1 DNA binding after exposure to normoxia (lanes 2–6), as reported previously (13). The rapid decrease of HIF-1 DNA binding correlated closely with the rate of decay of HIF-1α abundance (T1/2, <5 min) as demonstrated by Western blot analyses of the same cell extract preparations, whereas HIF-1β abundance remained unchanged when the same blot was reprobed with anti-HIF-1β (Fig. 2C, lanes 2–6).

To confirm that the disappearance of HIF-1α was not due to lack of HIF-1α mRNA, total RNA was prepared from cells treated under the same conditions as above and analyzed by RNase protection. Once again, HIF-1α mRNA levels were unaffected (Fig. 2A, lanes 2–6). Therefore, these results indicate that the HIF-1α protein is stabilized by hypoxia and decays very rapidly upon reoxygenation. To investigate whether normoxia directly destabilizes HIF-1, hypoxic extracts were incubated at room temperature, 21% O2, for 1, 5, 10, 20, or 40 min and loaded onto an EMSA gel at the respective time points. Results in Fig. 2D showed that HIF-1 binding activity persisted throughout the course, suggesting that the instability of HIF-1α under normoxic conditions depends on cell integrity.

To test whether oxygen-dependent regulation of the level of HIF-1α protein is due to alterations in translation, HIF-1 was activated in HeLa cells by incubation for a total of 3 h in 1% O2, and at 120, 150, and 165 min cycloheximide (final concentration, 100 μg/ml) was added anaerobically to separate plates in the chamber via a needle passed through a rubber stopper. No changes were noted at the 150- and 165-min time points, whereas at 120 min, this inhibitor of translation caused a modest reduction in the level of HIF-1α protein and in HIF-1 DNA binding (data not shown). These results indicate that in deoxygenated cells, HIF-1α protein cannot have a high turnover rate. Therefore, the rapid decay of HIF-1α protein seen with reoxygenation (Fig. 2C) cannot be due solely to suppression of HIF-1α translation. Importantly, this decay was not affected by cycloheximide and, therefore, does not depend upon ongoing protein synthesis.

The long 3' untranslated region of HIF-1α has been suggested to play a role in RNA stability (5). To confirm further that HIF-1α expression is controlled primarily at the protein level, full-length HIF-1α cDNA and HIF-1α cDNA lacking its 3' untranslated region were cloned into a eukaryotic expression vector downstream of the strong cytomegalovirus enhancer and promoter, designated pPHIF-1α and pPHIF-1α/HN. To achieve high level expression of exogenous HIF-1α, these constructs were transfected into 293 cells, which have a high degree of transfection efficiency, and analyzed by Western blot. As shown in Fig. 3, no significant amount of HIF-1α protein was detected under normoxic conditions (lanes 1, 3, and 5), whereas at low O2 tension, significant increases in HIF-1α protein were detected in cells transfected with either pHIF-1α or pHIF-1α/HN (lanes 4 and 6). Accumulation of HIF-1α protein was not much affected by the 3' untranslated region in HIF-1α mRNA. Moreover, the expression of endogenous HIF-1β was unaffected by overexpression of HIF-1α. These results show that oxygen decreases the levels of both overexpressed and endogenous HIF-1α, further supporting the conclusion that regulation is independent of transcription and protein synthesis.

H2O2 Blocks Accumulation of HIF-1α, Resulting in Inactivation of HIF-1—The exogenous addition of hydrogen peroxide has been shown to inhibit hypoxic induction of erythropoietin protein (21). As indicated in Fig. 4, we show that this is associated with suppression of Epo mRNA levels. After treatment with increasing concentrations of H2O2 for 15 min, Hep3B cells were exposed to 1% O2 for 8 h prior to analysis of Epo mRNA by ribonuclease protection. Epo mRNA expression was slightly inhibited by 0.1 mM H2O2 and was fully extinguished by 1 mM. This is a 3–5-fold higher concentration of peroxide than that generally required to elicit biological effects in cultured cells. In keeping with its suppression of the hypoxic induction of Epo mRNA, 1 mM H2O2 inhibited HIF-1 DNA binding in extracts prepared from hypoxic Hep3B cells (data not shown). As shown in Fig. 5A, top panel, exposure of HeLa cells to 1 mM H2O2 for 15 min, followed by incubation in 1% O2 for 2 h, also resulted in nearly complete abolition of HIF-1 binding. The specificity of this inhibition was demonstrated by incubating the same cell extracts with [32P]-labeled oligonucleotides containing an Oct-1 binding site and the heat shock element, respectively. As shown in Fig. 5A, pretreatment with hydrogen peroxide had no effect on Oct-1 DNA binding (middle panel), but as expected (17), induced binding of heat shock transcription factor to the heat shock element (bottom panel).

To elucidate the mechanism by which pretreatment with hydrogen peroxide inhibits HIF-1 DNA binding, levels of both...
HIF-1α and HIF-1β protein were quantified by Western blot analyses of these HeLa whole-cell extracts. The top panel of Fig. 5B shows that expression of HIF-1α protein was hypoxia-inducible, consistent with the results in Fig. 2C, but this induction was greatly inhibited by hydrogen peroxide pretreatment. In contrast, the abundance of HIF-1β protein was not significantly affected (bottom panel). To further determine the effect of hydrogen peroxide on the expression of HIF-1α, total RNA was isolated from cells treated under the same conditions as above and analyzed by ribonuclease protection. Despite the inhibition of HIF-1α mRNA protein accumulation, no significant changes in HIF-1α transcripts were observed (Fig. 5C).

To determine whether hydrogen peroxide has a direct effect on HIF-1 binding, hypoxic cell extracts were subjected to hydrogen peroxide in vitro prior to performing EMSAs. No loss of HIF-1 binding to DNA was observed at concentrations up to 1 mM (Fig. 5D), indicating that hydrogen peroxide acts upstream of HIF-1 activation.

As a test of whether hydrogen peroxide affects the translation process, in vitro translation of HIF-1α was performed in the presence and absence of hydrogen peroxide. Up to 10 mM H$_2$O$_2$ had no effect on the formation of translation product. Although this cell-free system departs substantially from in vivo conditions, this result suggests that hydrogen peroxide does not impact directly on HIF-1α synthesis but instead targets upstream event(s) in the sensing/signaling pathway, thereby blocking accumulation of the HIF-1α protein.

Redox Regulation Appears to Be Necessary for HIF-1 DNA Binding—Although, as demonstrated above, the binding of HIF-1α to DNA is primarily controlled at the level of HIF-1α accumulation, there is little information on what posttranslational modifications are necessary for this activation. The inhibitory effects of H$_2$O$_2$ on HIF-1 activity strongly suggest that redox chemistry is an important determinant of the stability of HIF-1α. Therefore, we examined the effects of sulfhydryl reagents on in vitro HIF-1 activity. Whereas N-ethylmaleimide (NEM) irreversibly modifies thiol groups, diamide promotes reversible disulfide bond formation (22, 23). In agreement with recently published results (24), when cell extracts were incubated with NEM or diamide prior to the addition to probe, HIF-1 DNA binding was significantly inhibited (Fig. 7, lanes 3 and 6) in a dose-dependent fashion (data not shown), suggest-

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**FIG. 4.** H$_2$O$_2$ inhibits Epo gene expression in a dose-dependent fashion. Hep3B cells were pretreated for 15 min with increasing amounts of hydrogen peroxide (final concentration, 0.1–1 mM) (lanes 3–6), followed by exposure to 1% O$_2$ for 8 h. Ribonuclease protection analysis was performed on 20 µg of total RNA. Protected Epo transcripts are indicated.

**Fig. 5.** Effects of hydrogen peroxide on HIF-1 DNA binding. In A, HeLa cells pretreated for 15 min with 1 mM hydrogen peroxide (lanes 2 and 4) were incubated under normoxic (lanes 1 and 2) or hypoxic (lanes 3 and 4) conditions for 2 h. Whole-cell extracts were prepared for EMSA using [32P]W18 (top panel), Oct-1 (middle panel), and HSE (bottom panel) oligonucleotides. In B, the same batch extracts were used for Western blot analysis using anti-HIF-1α (top) and anti-HIF-1β (bottom) antibodies. In C, following the same protocol, total RNA was extracted and analyzed by RNase protection using [32P]-labeled HIF-1α riboprobe. D, hypoxic extracts were incubated with 0.25–1 mM hydrogen peroxide (lanes 2–4) before addition to [32P]W18.

**FIG. 6.** The effect of hydrogen peroxide is reversible. HeLa cells were pretreated 1 mM H$_2$O$_2$ for 20 min and then subjected to hypoxia for 4 h (lane 3), or prior to hypoxia treatment, H$_2$O$_2$ was removed by rapidly rinsing cells twice with the medium (lane 4). Whole-cell extracts were analyzed by EMSA to examine HIF-1 binding (top panel) and by Western blotting to quantify the abundance of HIF-1α and HIF-1β (bottom panel). The middle panel shows an EMSA analysis of proteins binding to DR-2, a tandem repeat of hormone response elements on the 3' end of the Epo enhancer (49).
ing that sulfhydryl alkylation or oxidation prevents DNA binding. Consistent with the chemical properties of these reagents, subsequent addition of dithiothreitol (DTT) fully reversed the inhibitory effect of diamide (lane 7) but not that of NEM (lane 4). Moreover, the monothiol reducing agent, β-mercaptoethanol, showed the same effect as DTT (lane 8). To further demonstrate that thiol groups were the target of these reagents, cell extracts were first treated with diamide followed by NEM to test whether the thiol groups were still accessible to NEM after oxidation. As shown in Fig. 7, DTT, in the presence of NEM, still reversed the inhibitory effect of diamide (lane 12), indicating that thiol groups involved in HIF-1 activity had been fully oxidized by diamide, thereby preventing alkylation by NEM.

Since oxidation or alkylation of thiols prevents HIF-1 DNA binding and sulfhydryl reduction reversed the inhibitory effect of oxidation, we investigated whether sulfhydryl reductants could stimulate HIF-1 DNA binding. Contrary to the conclusions of others (24), we found that when oxidized thioredoxin purified from E. coli was added in the presence of DTT, a clear stimulation in HIF-1 DNA binding was reproducibly detected in hypoxic extracts (Fig. 8A, lane 4). Moreover, in the presence of DTT, thioredoxin not only reversed the inhibitory effect of diamide but also enhanced HIF-1 DNA binding over that in untreated extracts (compare lanes 5 with 7 and 8). In contrast, oxidized thioredoxin had no effect in the absence of DTT (data not shown).

To assay the functional effect of redox potential as a modulator of HIF-1 activity in intact cells, we transfected HeLa cells with a vector expressing human thioredoxin (pCMV-ADF) along with a luciferase reporter (pEpoE-luc) containing the human erythropoietin HIF-1 binding site. It has been shown that oxogenous expression of thioredoxin stimulates AP-1 activity but inhibits nuclear factor-κB (25, 26). As shown in Fig. 8B, overexpression of thioredoxin markedly potentiated the hypoxic induction of luciferase gene expression. Moreover, this effect correlated with the dose of vector (data not shown). Ref-1, a nuclear protein possessing both redox and apurinic endonuclease DNA repair activities (27, 28), has also been shown to facilitate AP-1 binding activity (29). When the Ref-1 expression vector (pCMV-APE) was cotransfected with the reporter, like thioredoxin, it further enhanced the hypoxic induction of the reporter (Fig. 8B). Moreover, when the HIF-1 binding site was mutated, no potentiation was observed with either thioredoxin or Ref-1. Thus, the effect of these proteins was HIF-1-dependent. Because of high redox buffering capacity of the cells, it is likely that both thioredoxin and Ref-1 were in the reduced form.

**DISCUSSION**

Diverse mechanisms have evolved to activate transcription factors in response to specific environmental signals. There is growing evidence that hypoxia regulates transcription of a broad repertoire of biologically important genes through a common mechanism of O₂ sensing, signal transduction, and transcription (30). The oxygen sensor is likely to be a heme protein (31, 32), perhaps a cytochrome b-like flavo-heme NAD(P)H oxidase that signals by altering intracellular peroxide and activated oxygen compounds (21, 33–35). Hypoxia results in the activation of heterodimeric HIF-1, enabling it to bind to cis elements of responsive genes. HIF-1α interacts specifically with p300/CBP, a transcriptional co-activator, thereby up-regulating gene expression (15).

Our studies address the mechanism responsible for HIF-1 activation by hypoxia. Others (5) have reported up-regulation of both HIF-1α and HIF-1β mRNA following exposure of Hep3B cells to hypoxia, cobalt, and desferrioxamine and rapid decay of both transcripts following transfer of cells from a hypoxic to a normoxic environment. However, the Northern blots in this report showed striking variability in both transcripts following transfer of cells from a hypoxic to a normoxic environment. Moreover, the pretreatment of cells with hydrogen...
Regulation of HIF-1 Activity

Regarded as one of the proteins most critical in the adaptive response of mammalian cells to hypoxia, HIF-1 is also a major mediator of tissue oxygen homeostasis. In the absence of oxygen, HIF-1 is rapidly degraded, whereas in hypoxic cells, allowing formation of the HIF-1α heterodimer and transcriptional activation of genes containing functional HIF-1 elements. Hydrogen peroxide can activate NF-κB when added to intact cells but has no effect on cell-free extracts (38). In both cases, the action of H₂O₂ is indirect, presumably via upstream signaling.

Although hydrogen peroxide has pleotropic effects on cell metabolism and function, impacting on various signaling pathways (39–41), including triggering of protein phosphorylation (42), its mechanism of action and that of reactive oxygen intermediates generated from peroxide depend on redox chemistry. In further support of the importance of the intracellular redox environment on the activation of HIF-1, we have shown that intracellular expression of either thioredoxin or a nuclear reducing protein Ref-1 up-regulates HIF-1-dependent hypoxic induction of a reporter gene (Fig. 8B). However, these experiments alter the redox status of the cell in an artificial manner. Physiologic signaling in a cell through a redox mechanism is likely to be channeled within the confines of specific subcellular compartments. Moreover, experimental maneuvers that affect upstream signaling in a redox-dependent manner may have no relevance to direct modification of HIF-1 by sulfhydryl reagents. Again, nuclear factor-κB may be an apt analogy; peroxide enhances its activation via upstream signaling steps involving release from its inhibitor (25, 26, 38, 43), whereas direct modification of nuclear factor-κB by sulfhydryl reagents inhibits DNA binding (44). Our experiments suggest the need for detailed structure-function studies of HIF-1α to ascertain whether, like Fos/Jun (45), USF (46), Rel/kB (47), and bovine papilloma type 1 E2 protein (48), reduction of critical cysteine sulfhydryls plays an important functional role.

Taken together, as depicted in Fig. 9, our results indicate that the pathway leading from the sensing of hypoxia to the activation of HIF-1 is critically dependent on the relative abundance of its α subunit. The striking difference in the oxygen-dependent expression of HIF-1α and HIF-1β at the protein level is consonant with the apparent specificity of HIF-1α, the only known function of which is in hypoxia-specific regulation, whereas HIF-1β can heterodimerize with the aryl hydrocarbon receptor and perhaps with other transcription factors. The effect of oxygen in acutely lowering levels of HIF-1α is mimicked by hydrogen peroxide, a finding which is consistent with a large body of experimental work implicating reactive oxygen intermediates in the signaling process (30). We present both in vitro and in vivo experiments that indicate that redox chemistry contributes importantly to HIF-1 activation.

Acknowledgments—We are grateful to Markus R. Probst and Oliver Hankinson for anti-ARNT antisera; Greg Semenza for the HIF-1α cDNA clone; and Patrick Baeuerle, Lynn Harrison, and Bruce Demple for constructs. We thank Ben Ebert and Mark Goldberg for helpful suggestions.

Note Added in Proof—Recently, Wood et al. (50) and Grardin et al. (51) have also reported that HIF-1α mRNA and HIF-1β mRNA are both constitutively expressed and not significantly up-regulated by hypoxia.

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