A Redox Mechanism Controls Differential DNA Binding Activities of Hypoxia-inducible Factor (HIF) 1α and the HIF-like Factor*

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Hypoxia-inducible factor 1α (HIF-1α) and the HIF-like factor (HLF) are two highly related basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) homology transcription factors that undergo dramatically increased function at low oxygen levels. Despite strong similarities in their activation mechanisms (e.g. they both undergo rapid hypoxia-induced protein stabilization, bind identical target DNA sequences, and induce synthetic responses), they are both essential for embryo survival via distinct functions during vascularization (HIF-1α) or catecholamine production (HLF).

It is currently unknown how such specificity of action is achieved. We report here that DNA binding by HLF, but not by HIF-1α, is dependent upon reducing redox conditions. In vitro DNA binding and mammalian two-hybrid assays showed that a unique cysteine in the DNA-binding basic region of HLF is a target for the reducing activity of redox factor Ref-1. Although the N-terminal DNA-binding domain of HIF-1α can function in the absence of Ref-1, we found that the C-terminal region containing the transactivation domain requires Ref-1 for full activity. Our data reveal that the hypoxia-inducible factors are subject to complex redox control mechanisms that can target discrete regions of the proteins and are the first to establish a discriminating control mechanism for differential regulation of HIF-1α and HLF activity.

Cells of aerobic organisms depend upon a plentiful supply of oxygen for energy production, and consequently, a number of mechanisms have evolved to sense and respond to disruptions of oxygen homeostasis. Although several signal transduction and gene regulatory mechanisms have been established to operate in response to oxidative stresses induced by intracellular oxygen radical production or cell penetration by oxidants, mammalian gene regulatory pathways that respond to hypoxia (low oxygen) have only recently begun to be understood. Analysis of hypoxia-induced activation of the erythropoietin gene led to the cloning of HIF-1α 1 (1), a transcription factor that exhibits marked increases in stability and transcription po-

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† The abbreviations used are: HIF-1α, hypoxia-inducible factor 1α; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; HLF, HIF-like factor; EPO, erythropoietin; CREB, cAMP response element-binding protein; NF-κB, nuclear factor κB; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; XRE, xenobiotic response element; HRE, hypoxia response element; DR, dioxin receptor; GR, glucocorticoid receptor.

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FIG. 1. Bacterial expression and purification of the GST fusion proteins containing truncated HIF-1α-(1–245) or HLF-(1–265) full-length ARNT. A and B, the HIF-1α-(1–245) and HLF-(1–265) fusion proteins were affinity-purified from IPTG-induced culture (lane 2) on glutathione-Sepharose, and 500 ng was analyzed (lane 3) by 10% SDS-PAGE and Coomassie Blue staining. C, the glutathione-Sepharose-purified Arnt fusion protein was subjected to Western analysis. A total of 20 μl of affinity-purified Arnt eluant (lane 3) from IPTG-induced culture was resolved by 7.5% SDS-PAGE and immunooblotted with anti-Arnt rabbit polyclonal antisera. The solid bars represent molecular mass standards (in kilodaltons), and the arrows indicate the positions of the GST fusion proteins.

DNA-binding domains have been found to have their DNA binding activities controlled by reducing factors such as Ref-1 (e.g., CREB, NF-κB, and Myb (12); p53 (13); and Pax5 and Pax8 (14, 15)) or thioredoxin (16). We report here that Ref-1 imparts DNA binding to the bHLH domain. Mammalian two-hybrid assays, and the asterisk denotes free probe.

is important for HIF-1α activity in a region C-terminal to the bHLH domain. Thus, the activities of both HIF-1α and HLF are under complex modes of redox control, with some distinction between domains subject to redox regulation, providing the first instance of differential control for activation of these two proteins. These results suggest a novel mechanism whereby specificity of hypoxia-induced gene regulation may be achieved.

EXPERIMENTAL PROCEDURES

Bacterial Expression and Purification of bHLH/PAS Proteins—The generation of the GST-tagged expression plasmids pDR-(1–287)/GEX4T3 and pArnt/GEX4T3 has been described previously (18). pHIF-1α-(1–245)/GEX4T3 was generated by inserting an NcoI/SalI fragment from pHIF-1α/Bs (19) into NcoI/Xhol-digested pArnt-(1–140)/GEX4T3 (18), thereby replacing Arnt coding sequence with codons 1–245 of human HIF-1α. pHLF-(1–265)/GEX4T3 was generated by insertion of a BamHI/EcoRI-digested polymerase chain reaction fragment spanning codons 1–265 of murine HLF cDNA (4) into BamHI/EcoRI-digested pGEX4T3. HIF-1α-(1–245)/GEX4T3 was created by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s directions. The fidelity of the polymerase chain reaction-assisted cloning and generation of the mutant form of HIF-1α-(1–245) was verified by dideoxy sequencing. pGEX4T3 plasmids in *Escherichia coli* strain BL21 (LYs) were grown in LB broth supplemented with 2% glucose and induced with 1 mM IPTG. Harvested cells were resuspended in Buffer A (20 mM Tris (pH 7.6) and 137 mM NaCl) containing 0.1% Tween 20, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, and 5% glycerol) and lysed by sonication. The lysate was centrifuged at 7500 rpm for 15 min at 4 °C. The supernatant was then loaded onto a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) and washed once with Buffer A containing 0.1% Tween 20 and once without Tween 20. GST-tagged fusion proteins were eluted with 10 mM reduced glutathione. The purity of the isolated protein was analyzed by SDS-PAGE, and protein concentration was determined by the Bradford assay (BioRad).

Purification of His-tagged Ref-1(1–190)—*E. coli* strain M15 with the pRef1-(1–190)/QEx30 plasmid (a kind gift from S. Xanthoudakis and T. Curran, St. Jude Children’s Research Hospital) (20) was grown in LB broth and induced with 1 mM IPTG. Harvested cells were resuspended in Buffer B (8.0 mM urea, 20 mM Tris (pH 8.0), 300 mM NaCl, and 10% glycerol) and lysed by two cycles of freeze (–80 °C)/thawing. The lysate was centrifuged at 7000 rpm for 15 min at 4 °C. The supernatant was applied to a nickel-nitrilotriacetic acid column (QIAGEN Inc.) and washed with Buffer B. The bound His-tagged protein was then eluted by lowering the pH using Buffer C (8.0 mM urea, 20 mM Tris (pH 5.0), 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, and 5% glycerol) with a 6.0 to 0.0 mM urea stepwise reducing gradient. Protein purity was ana-
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Electrophoretic Mobility Shift Assay (EMSA)—Annulled double-stranded oligonucleotides were end-filled with \(^{32}P\)-labeled dNTPs using Klenow enzyme and used as probes for gel shift assays. The W18 probe contains the hypoxia response motif from the erythropoietin enhancer (21). The XRE is a probe containing a dioxin receptor response motif from the cytochrome P450IA1 gene (18). Gel mobility shift assays were carried out in a 20-μl volume with the bacterially expressed and purified protein HIF-1α-(1–245), HLF-(1–265), or DR-(1–265) with the partner factor Arnt as specified in a two-step process. Briefly, protein samples (100–400 ng each) in the presence or absence of the reducing agent Ref-(1–190) or DTT were allowed to dimerize and interact at room temperature for 30 min. Binding buffer (final concentrations: 10 mM Tris (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM MgCl₂, 50 mM KCl, 50 mM NaCl, 0.1 mM DTT, 50 ng/μl poly(dI·dC), and 0.5 μg/μl bovine serum albumin) and the radiolabeled probe W18 or XRE (>10,000 cpm) were then added, and the reactions were transferred to 4 °C and incubated for an additional 30 min. Protein-bound DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel run in 25 mM Tris/glycine (pH 8.0) and 0.1 mM EDTA at 4 °C.

Transient Transfections—The luciferase reporter plasmid pHER-Luc (4) and pXRE-Luc (22) and the expression plasmid pArnt/CMV4 (23) have been described previously. The pHLF/EF-bos expression plasmid (8) was constructed by initially shuttling an XhoI/NotI fragment from the pHRE-Luc probe (4) into NotI/AatII-digested SmaI301 vector (Invitrogen) to generate pHER/LSL301. A BglII/NotI fragment from pHRE/LSL301 was then subcloned into BamHI/NotI-digested pEF-bos-cs eukaryotic expression plasmid (24). The pHIF-1α/EF-bos expression plasmid was constructed by subcloning a SnmaU2XmaI fragment from pHIF-1α/CMV4 (19) into EcoRV/XhoI-digested pEF-bos-cs. The DR bHLH/HIF-1α chimeric cDNA was generated by joining polymerase chain reaction fragments containing the mouse dioxin receptor bHLH coding region (codons 1–83) to the coding region 3′ of the human HIF-1α bHLH (codons 74–826) via an XhoI linker and subcloning into pCMV4. HeLa Tet-on cells (CLONTECH) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

For transient transfections HeLa Tet-on cells were plated onto 24-well plates at 5.0 × 10⁵ cells/well. After 24 h, transfections were carried out using the liposomal transfection reagent DOTAP (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Each transfection contained 100 ng/well reporter plasmid, 25 ng/well Renilla internal luciferase control (Promega), 2.0 μg/well pET-PetePicky-cRef-1, and 100 ng/well each expression plasmid or empty expression vector as specified and was performed in duplicate. At the time of transfection, one well was treated with a 1 μg/ml concentration of the tetracycline analog doxycycline (Sigma). After treatment (48 h), all cells were washed and harvested in phosphate-buffered saline and then lysed with dual luciferase lysis buffer, and extracts were analyzed by dual luciferase reporter assay (Promega).

Mammalian Two-hybrid Assay—pCMX/Ref-1/VP16 was generated by inserting a BamHI/NheI-digested polymerase chain reaction fragment of Ref-1 into a BamHI/NheI-digested pCMVX/VP16 vector. COS-1 cells were seeded onto six-well plates (3 cm) and transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol. Briefly, 500 ng of a Gal4-luciferase reporter (18) was cotransfected together with 10 ng of the corresponding Gal4 chimeric expression plasmid and 1 ng of the Ref-1/VP16 fusion construct. Cells were harvested 48 h after transfection, and luciferase activity was determined using the Gene Glow assay system (BioThema).

RESULTS

Differential DNA Binding Activity of HIF-1α and HLF—We have previously noted that the PAS A region of the dioxin receptor is important for the DNA binding activity of the DR/Arnt heterodimer (18). As an extension of these studies, we sought to analyze the influence of the PAS A domain on the DNA binding activity of two other bHLH/PAS proteins, the closely related hypoxia-inducible factors HIF-1α and HLF. N-terminal regions of HIF-1α and HLF containing the bHLH domains, the PAS A domains, and the intervening amino acids between PAS A and PAS B (termed HIF-1α-(1–245) and HLF-(1–265) respectively) were expressed as GST fusion proteins in bacteria. A GST fusion protein of the full-length partner protein Arnt was also expressed in bacteria, and all proteins were purified to near homogeneity by glutathione-Sepharose affinity chromatography (Fig. 1). Electrophoretic mobility shift assays have previously shown that in vitro translated HIF-1α and HLF, when combined with Arnt, recognize the same DNA sequences from hypoxia-responsive regions in the EPO and vascular endothelial growth factor genes (4, 5). As expected, when tested in isolation, bacterially expressed HIF-1α, HLF, and Arnt had no affinity for the hypoxia response element from the EPO gene (Fig. 2, lanes 2–4). Surprisingly, however, we found that a mixture of purified HLF and Arnt showed barely detectable DNA binding activity on the EPO hypoxia response element (Fig. 2, lanes 5–7), whereas equivalent quantities of HIF-1α and Arnt exhibited clear DNA binding (lanes 8–10). This striking difference in activity was unexpected as sequence analysis of HLF and HIF-1α revealed a strong homology between the two DNA-binding and dimerization regions, with 83% identity between the bHLH domains and just under 70% identity over the PAS domains. These results stimulated us to investigate whether other factors might invoke differential control over DNA binding activity between HIF-1α and HLF.

Redox Factor Ref-1 Stimulates DNA Binding of the HLF/Arnt Heterodimer, but Does Not Influence HIF-1α/Arnt DNA Binding—Upon closer inspection of the amino acid sequences for HIF-1α and HLF, it was noticed that the only difference within a 13-amino acid stretch of the DNA-binding basic regions between the two proteins was that cysteine 25 in HLF correlated with serine 28 in HIF-1α. This single amino acid difference is conserved for both human and mouse proteins. (Despite the original published sequence of HLF describing amino acid 25 as a serine, resequencing has confirmed a cysteine at this position, consistent with EPAS1 (5), HIF-related factor (6), and MOP2 (7) sequences.) The position of this difference was intriguing, as cysteines in the DNA-binding basic regions of the oncoproteins Fos and Jun were found to mediate susceptibility for redox control of DNA binding activity (25). Strikingly, these cysteines are in a highly similar immediate environment, flanked by basic amino acids (Fig. 3). In the case of Fos and Jun, mutation of the conserved cysteines to serines provided a higher affinity DNA-binding complex that was resistant to redox control and displayed increased cell transforming ability (26). Xanthoudakis and Curran (12) isolated a protein from cell extracts (Ref-1) that was necessary for strong DNA binding activity of Fos and Jun. Ref-1 was found to be identical to the DNA repair enzyme APE/HAP-1 and to harbor two distinct activities, an N-terminal reducing function capable of interacting with cysteines and a C-terminal DNA repair function (20). To test whether the redox factor Ref-1 could influence DNA binding of HIF-1α and HLF, we expressed a histidine-tagged N-terminal redox domain of Ref-1 in bacteria and purified the protein by nickel affinity chromatography (Fig. 4A). Upon addition of this purified Ref-1 (1–190) polypeptide to the EMSA
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Serine-to-Cysteine Point Mutation Renders DNA Binding of HIF-1α Susceptible to Redox Control—The similarities of redox control for DNA binding activities in the Fos/Jun heterodimer and the HLF/Arnt heterodimer suggest that the cysteine in the basic region of HLF is a likely target for redox control. However, other cysteines in the HLF region being analyzed might also be affected by the redox conditions of the experiment. We therefore sought to obtain direct evidence that the basic region Cys25 is the target for redox control of HLF DNA binding. To this end, we created a point mutant in the HIF-1α-(1–245) N-terminal sequence that resulted in a switch from Ser28 to Cys28. This point mutant polypeptide was expressed as a GST fusion protein in bacteria and purified (Fig. 5A). When incubated with radiolabeled EPO hypoxia response element, neither HIF-1α-(1–245) nor HIF-1α-(1–245) S28C alone showed any DNA binding activity (Fig. 6B, lanes 3 and 4). When combined with bacterially expressed and purified Arnt, HIF-1α-(1–245), but not HIF-1α-(1–245) S28C, exhibited DNA binding in the absence of reducing agents (Fig. 6B, compare lanes 6 and 9). Strikingly, while the addition of either DTT or purified Ref-(1–245) alone showed any DNA binding activity for the HLF/Arnt heterodimer, the addition of dithiothreitol to the protein/DNA incubation also allowed conversion of the HLF/Arnt heterodimer to a DNA-binding form (Fig. 4B, lane 6).

The Ref-(1–190) redox domain stimulates the DNA binding activity of the HLF-(1–265)/Arnt heterodimer, but not that of the HIF-1α-(1–245)/Arnt heterodimer. A, bacterial expression and purification of recombinant hexahistidine-tagged Ref-(1–190). Ref-(1–190) was nickel affinity-purified from IPTG-induced culture (lane 2), and 5 μg (lane 3), 2.5 μg (lane 4), and 1 μg (lane 5) were analyzed by 15% SDS-PAGE and Coomassie Blue staining. The solid bars represent molecular mass standards (in kilodaltons), and the arrow indicates the position of the Ref-(1–190) protein. B and C, the effects of Ref-(1–190) and DTT on the DNA binding activity of HLF-(1–265) and HIF-1α-(1–245). One-hundred nanograms of HLF-(1–265) or HIF-1α-(1–245) was incubated with 2 μl of Eluant eluant (B, lanes 5–10; and C, lanes 5–9) and assayed for DNA binding activity following no treatment (lane 5) or treatment with 10 mM DTT (lane 6), increasing amounts of Ref-(1–190) (100, 200, and 400 ng (lanes 7–9, respectively), or 4 μl of Ref-1 storage buffer (lane 10). DNA binding activities were measured using gel mobility shift assays with the 32P-labeled W18 probe. Lane 1, probe alone; lanes 2–4, isolated samples of either Arnt eluant (2 μl), 100 ng of HLF-(1–265), 100 ng of HIF-1α-(1–245), or 400 ng of Ref-(1–190) as indicated. The arrow indicates the position of HRE retarded complexes, and the asterisk denotes free probe.
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190) did not influence the DNA binding activity of the HIF-1α-(1–245) complex, both reducing agents were able to invoke strong DNA binding of the HIF-1α-(1–245) S28C complex (Fig. 6B, compare lanes 6–8 with lanes 9–12). The ability to convert the redox-resistant DNA binding activity of HIF-1α into the redox-sensitive DNA binding activity seen analogous to that of HLF establishes that placing a cysteine in the DNA-binding basic region of these bHLH/PAS proteins renders them susceptible to redox control. Moreover, a dose dependence for the influence of Ref-1 on the DNA binding activity of HIF-1α-(1–245) S28C was observed (Fig. 6C, lanes 7–12), consistent with Ref-1 reducing HIF-1α-(1–245) S28C through protein/protein interaction.

Ref-1 Interacts with the bHLH N Terminus of HLF, but Not with That of HIF-1α—The above results are consistent with a model in which Ref-1 provides a reducing activity important for HLF DNA binding, whereas HIF-1α DNA binding is impervious to redox control. To obtain direct evidence that Ref-1 is capable of intracellular interaction with the HLF N-terminal bHLH/PAS A domain, we performed a mammalian two-hybrid assay. Fusion proteins containing either the HLF-(1–245) domain or the HIF-1α-(1–245) domain attached to the DNA-binding domain of Gal4 were coexpressed in COS-1 cells with a fusion protein consisting of Ref-1 attached to the VP16 transactivation domain. The activity of a luciferase reporter gene containing Gal4 response elements was unaffected by expression of the Ref-1/VP16 fusion protein alone, whereas individual expression of Gal4/HIF-(1–265) or Gal4/HIF-1α-(1–245) provided the same degree of activity on the reporter gene (Fig. 7). As the N-terminal regions of HLF and HIF-1α lack transactivation domains (27), the increased reporter gene activity seen for these constructs may be due to the recruitment of cellular Arnt, which is known to harbor a strong transactivation domain, via the HLH regions of the chimeras. Coexpression of Ref-1/VP16 with Gal4/HIF-1α-(1–245) produced a mild decrease in reporter gene activity compared with expression of Gal4/HIF-1α-(1–245) alone, which is in stark contrast to the increase in activity seen for the Gal4/HLF-(1–265) construct upon coexpression of Ref-1/VP16 (Fig. 7). These results provide strong evidence that Ref-1 can interact with the HLF N-terminal region within cells, but not with the HIF-1α N terminus, and are in excellent agreement with the in vitro DNA binding assays showing that Ref-1 is important for the DNA binding activity of the HLF bHLH/PAS A region, but not the HIF-1α bHLH/PAS A region.

Antisense Ref-1 Decreases the Transcriptional Activity of Both HLF and HIF-1α—Ref-1 is a ubiquitous protein that has recently been reported to influence DNA binding of several transcription factors, including AP-1, Myb, and CREB (28) and Pax5 and Pax6 (14). Our results demonstrating the strong influence of Ref-1 on DNA binding of HLF predict that the ability of HLF to induce transcription would be dependent upon sufficient cellular levels of Ref-1. To investigate whether HLF activity on a reporter gene would be compromised in a limiting Ref-1 environment, we used a tetracycline-inducible vector to express antisense Ref-1 RNA during a transient transfection experiment. HeLa Tet-on cells stably express a modified version of the bacterial tetracycline repressor protein, which, in the presence of tetracycline, activates promoters containing tet operator sequences (29). The coding sequence of Ref-1 was subcloned in reverse direction into a tet-responsive vector so as to express antisense Ref-1 transcript in the presence of tetracycline in HeLa Tet-on cells. The antisense Ref-1 vector was cotransfected into HeLa Tet-on cells with expression vectors for HLF and Arnt and a luciferase reporter gene containing four copies of the hypoxia response element from the EPO gene. Coexpression of HLF and Arnt produced an ∼25-fold increase in reporter gene activity over basal levels of the reporter gene alone, whereas in the presence of antisense Ref-1, this increase dropped to ∼8-fold (Fig. 8A). In addition, expression of antisense Ref-1 decreased the activity of HIF-1α in an identical reporter assay, implying that Ref-1 is also important for full activity of the HIF-1α/Arnt heterodimer. Expression of antisense Ref-1 also decreased basal levels of the HRE-luciferase reporter gene, presumably due to decreased activity of endogenous HLF and HIF-1α proteins, which have both been re-

FIG. 5. DNA binding activity of the DR-(1–287)/Arnt heterodimer is not stimulated by Ref-(1–190). A, bacterial expression and purification of the truncated DR-(1–287)/GST fusion protein. The DR-(1–287) fusion protein was affinity-purified from IPTG-induced culture (lane 2) on glutathione-Sepharose, and 400 ng (lane 3) was analyzed by 10% SDS-PAGE and Coomassie Blue staining. The solid bars represent molecular mass standards (in kilodaltons), and the arrow indicates the position of the DR-(1–287) fusion protein. B, the effects of Ref-(1–190) on the DNA binding activity of DR-(1–287). One-hundred nanograms of DR-(1–287) and 2 μl of Arnt eluant (lanes 5–9) were assayed for DNA binding activity following no treatment (lane 5) and after incubations with 10 mM DTT (lane 6) or increasing amounts of Ref-(1–190) (100, 200, and 400 ng (lanes 7–9, respectively)). DNA binding activities were measured using gel mobility shift assays with the 32P-labeled XRE probe. Lane 1, probe alone; lanes 2–4, isolated samples of Arnt eluant (2 μl), 100 ng of DR-(1–287), and 400 ng of Ref-(1–190), respectively. The arrow indicates the position of XRE retarded complexes, and the asterisk denotes free probe.
ported to be expressed in HeLa cells during normoxia (8). The decreased reporter activities seen in the presence of antisense Ref-1 were not due to nonspecific detrimental influences on transcription, as a control Rous sarcoma virus-luciferase reporter gene was unaffected by expression of antisense Ref-1 (Fig. 8B).

Ref-1 Is Required for the Activity of the HIF-1α C Terminus—Expression of antisense Ref-1 decreases the ability of HIF-1α to function in a reporter gene assay, although our DNA binding assays revealed that Ref-1 does not influence the DNA binding activity of the HIF-1α/Arnt heterodimer. One interpretation of these results is that Ref-1 is needed for the activity of the HIF-1α C-terminal region. Two transactivation domains exist in the C terminus, separated by an inhibitory region (27, 30, 31).
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Figure 7. Ref-1 specifically interacts with the bHLH/PAS A motif of HLF. The intracellular interaction of Ref-1 with the bHLH/PAS A motif of HLF and HIF-1α was monitored using mammalian two-hybrid assays. COS-1 cells were cotransfected with 500 ng of Gal4-luciferase reporter and 10 ng of expression vectors chimeric for the DNA-binding domain (DBD) of Gal4 with HLF-(1–265) or HIF-1α-(1–245) and 1 ng of Ref-1/VP16 as specified. After 48 h, cells were assayed for luciferase activity, and the results are depicted as -fold induction over reporter gene activity in the presence of the non-chimeric DNA-binding domain of Gal4 with one representative experiment shown. TK, thymidine kinase.

The previous reports detailing the influence of oxidants on hypoxia-induced activities have interpreted their data only in terms of the effects on HIF-1α. Whereas it is apparent that strong oxidizing conditions will prevent HIF-1α activity, our results suggest that subtle changes in redox conditions may play a distinct role in determining the relative activities of HIF-1α and HLF. This possibility is due to the cysteine in the DNA-binding domain of HLF being surrounded by basic residues, an environment that has been found to lower the pK_a of cysteine thiols and to render them extremely susceptible to oxidation (37, 38). During weak or transient increases in redox potential, the HLF protein may therefore be prevented from forming a DNA-binding entity, whereas HIF-1α would not suffer any down-regulatory effects. Notably, transient low level release of reactive oxygen species such as _H_2O_2_, _O_2¬ and -OH (39, 40) or milder signaling oxidants such as NO (41) have been shown to oxidize cysteine sulphydryl groups to disulfides and sulfinic acids, reversible modifications that have been proposed to be employed during redox-initiated signal transduction processes (39).

**Redox Control of Eukaryotic Transcription Factors**—Although both cytoplasmic and nuclear compartments of the cell are generally considered reducing environments, there is mounting evidence to suggest that oxidized forms of some proteins can exist _in vivo_ and are subject to activation by redox signaling pathways. For example, E2A transcription factors have been found to form an intermolecular disulfide bond in B-lymphocyte cells, stabilizing homodimers to affect DNA binding. In other cell types such as muscle, reducing activities prevent E2A homodimerization and promote heterodimeric complexes with MyoD or Id (42). The Pax8 paired domain protein, a transcription factor important for thyroid development that also contains critical cysteines that require Ref-1 for full DNA binding activity, has been reported to exist predominantly in an oxidized intracellular form when Ref-1 levels are low, but in reduced form when Ref-1 levels are high (14). The potential _in vivo_ significance of Ref-1 control of Pax8 DNA binding activity is strengthened by the observation that thyrotropin stimulates mRNA and protein expression for both Pax8 and Ref-1 (14, 43). It is particularly notable that hypoxia has been reported to increase Ref-1 mRNA and protein levels (44), consistent with a role for Ref-1 in the hypoxic response. Taken together, these data suggest that during normal cellular metabolism, some eukaryotic transcription factors harbor cysteines that can readily exchange between oxidized and reduced states, a reversible switching mechanism demonstrated to be highly successful in controlling the activity of the prokaryotic transcription factor OxyR (45) and, more recently, the stress-activated chaperone hsp33 (46).

To date, eukaryotic transcription factors have been predominantly found to require reduced forms for full activity. These
include Fos and Jun, CREB, ATF-1 and -2, Myb, PEBP2/CFB (12); p53 (13); Pax proteins (14); and NF-Y (47), all of which have their DNA binding activities dependent upon, or increased by, Ref-1. In a similar fashion, the functions of NF-κB and the glucocorticoid receptor are positively influenced by thioredoxin. Intriguingly, protein/protein interaction between thioredoxin and Ref-1 has been demonstrated in mammalian two-hybrid assay (48), and the solution structure of the interaction has been solved (49). As thioredoxin is cytoplasmic and Ref-1 is nuclear in most cell types, a redox cascade is now thought to exist where Trx moves to the nucleus in response to cellular stress to activate Ref-1 and to enhance the activity of substrate transcription factors (48). Indeed, hypoxia treatment has recently been shown to translocate Trx from the cytoplasm to the nucleus (60).

We have presented data here to show that Ref-1 is important for the activities of the HIF-1α C terminus (Fig. 9) and the HLF DNA-binding domain. The close similarity between the HLF and HIF-1α C-terminal transactivation domains implies that Ref-1 might also affect the activity of the HLF C-terminal region, which in fact has very recently been observed during analysis of HLF chimeric proteins (60). A potential Ref-1 target cysteine has been suggested to exist in the C termini of both HIF-1α and HLF (60). Thus, a complex and multifaceted mechanism of redox control over hypoxia-inducible gene expression exists, with reducing activities being important for both DNA binding and transactivation of HLF and for transactivation, but not DNA binding, of HIF-1α (Fig. 10). Multiple mechanisms of redox control over function of transcription factors are not without precedent, as recent analyses of the glucocorticoid receptor have shown that activities such as ligand binding (16), DNA binding (17), and nuclear translocation (52) are all subject to redox control. Redox control of the nuclear localization of the GR involves a cysteine in the nuclear localization signal that is preceded by two basic residues, again consistent with the notion that such a cysteine would be particularly susceptible to oxidation. Indeed, mild oxidizing conditions were enough to inhibit nuclear translocation of the GR, whereas mutation of
the critical cysteine to a serine abrogated this inhibition (52). Moreover, nuclear translocation of the GR showed varied sensitivities to H2O2 in different cell types, with GR activity on reporter genes being affected by physiological concentrations of H2O2 (20–100 mM) are required to disrupt ligand binding (54), implying that separate receptor activities are subject to differential redox control. As HIF-1α has recently been shown to undergo hypoxia-induced nuclear translocation (55), it will be interesting to investigate whether this process is influenced by redox conditions.

Our data showing redox dependence of DNA binding for HLF, but not for HIF-1α, represent the first differential control mechanism for these factors. It is proposed that HLF and HIF-1α have distinct target genes such as the HLF-specific Tie2 receptor tyrosine kinase (5) and the vascular endothelial growth factor receptor Flk-1 (61) as well as differential activity on common target genes, e.g. vascular endothelial growth factor (5). To understand the disparate and essential functions of HLF and HIF-1α in the developing embryo, it will be of major importance to fully decipher differential activation and gene regulatory mechanisms between these two factors. The realization that a redox mechanism can invoke specificity for DNA binding activity between HLF and HIF-1α is an initial step toward this goal. A reversible redox-dependent switch for DNA binding may also operate in paired domain proteins, which contain two DNA-binding regions (the PAI and RED domains) that recognize different DNA sequences and that can function either independently or in concert. DNA binding of the PAI domain is abrogated by oxidation, whereas the RED domain remains unaffected, creating a situation in which a change in redox conditions may switch promoter binding affinity or specificity (15). Although post-translational control of protein activity is commonly understood in terms of phosphorylation status, it is becoming increasingly apparent that other less characterized post-translational modifications such as those affected by redox factors (56, 57), acetylation (58), or immunophilins with prolyl isomerase activity (59) can play important regulatory roles in the critical processes of cell signaling and transcriptional control.
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