Role of Prolyl Hydroxylation in Oncogenically Stabilized Hypoxia-inducible Factor-1α*

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Stabilization of the hypoxia-inducible factor-1 (HIF-1) protein is essential for its role as a regulator of gene expression under low oxygen conditions. Here, employing a novel hydroxylation-specific antibody, we directly show that proline 564 of HIF-1α and proline 531 of HIF-2α are hydroxylated under normoxia. Importantly, HIF-1α Pro-564 and HIF-2α Pro-531 hydroxylation is diminished with the treatment of hypoxia, cobalt chloride, desferrioxamine, or dimethyloxalyglycine, regardless of the E3 ubiquitin ligase activity of the von Hippel-Lindau (VHL) tumor suppressor gene. Furthermore, in VHL-deficient cells, HIF-1α Pro-564 and HIF-2α Pro-531 had detectable amounts of hydroxylation following transition to hypoxia, indicating that the post-translational modification is not reversible. The introduction of v-Src or RasV12 oncogenes resulted in the stabilization of normoxic HIF-1α and the loss of hydroxylated Pro-564, demonstrating that oncogene-induced stabilization of HIF-1α is signaled through the inhibition of prolyl hydroxylation. Conversely, a constitutively active Akt oncogene stabilized HIF-1α under normoxia independently of prolyl hydroxylation, suggesting an alternative mechanism for HIF-1α stabilization. Thus, these results indicate distinct pathways for HIF-1α stabilization by different oncogenes. More importantly, these findings link oncogenesis with normoxic HIF-1α expression through prolyl hydroxylation.

Conditions of low oxygen tension, or hypoxia, play important roles in normal physiological processes as well as in tumorigenesis. Tumor cell expansion requires new blood vessel formation (angiogenesis) to supply oxygen and nutrients. However, tumor blood vessels tend to be highly irregular and malformed. Consequently, solid tumors are subjected to transient changes in oxygenation due to the unpredictable opening and closing of malformed vessels. These areas of lowered oxygen concentration reduce the sensitivity of the tumor to radiation and some anticancer drugs. Radiation therapy specifically requires molecular oxygen to form cytotoxic DNA double strand breaks, whereas chemotherapeutic agents require access to proliferating cells to induce cytotoxicity. In addition, hypoxia can select for expansion of tumor cells that have diminished apoptotic sensitivity to radiotherapy or chemotherapy.

Thus, hypoxia promotes both malignant progression and reduces sensitivity to radiotherapy and chemotherapy.

Under hypoxic conditions, the expression of glycolytic enzymes is increased to compensate for the decreased respiratory generation of ATP (2). Analysis of the enhancer elements of many hypoxia-induced genes involved in glycolysis reveals a common hypoxia-responsive element (HRE)1 with a core sequence of 5’-RCGTG-3’. The transcription factor that recognizes this HRE has been identified as the hypoxia-inducible factor-1 (HIF-1), a member of the basic helix-loop-helix Per, AhR, and Sim (bHLH-PAS) family of transcription factors (3, 4). HIF-1 is composed of an α-subunit, which is degraded under normoxic conditions but stabilized under hypoxic conditions, and a constitutive β-subunit. This heterodimer is able to bind target DNA sequences to initiate gene transcription.

Stabilization of HIF-1 occurs through the inhibition of 4-prolyl hydroxylase activity, an enzyme that requires molecular oxygen to be functional (5). This enzyme has been proposed to be a critical oxygen sensor and regulator of the HIF-1 transcriptional response to low oxygen conditions. Hydroxylation of HIF-1α decreases protein stability under normoxic conditions (6, 7). A conserved proline residue, proline 564, is hydroxylated under normoxia, allowing the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex to bind HIF-1α (8, 9). The VHL complex adds ubiquitin to HIF-1α, which is then degraded by the proteasome. Under hypoxic conditions, it is proposed that 4-prolyl hydroxylase activity decreases because of a lack of oxygen, resulting in diminished proline 564 hydroxylation and HIF-1α protein stabilization.

Initial studies of HIF-1α hydroxylation involved labor-intensive in vitro assays (5–7, 10, 11). The hydroxylation event was first identified through the use of recombinant fusion proteins that were produced in Escherichia coli and treated with rabbit reticulocyte lysates. Fusion proteins treated with reticulocyte lysates interacted with VHL, whereas untreated fusion proteins did not bind VHL. Binding assays of in vitro translated HIF-1α and VHL indicated that only HIF-1α treated with unprocessed rabbit reticulocyte lysate was able to bind VHL. Most importantly, peptides of HIF-1α in which residue 564 was changed from a proline to a glycine could not abrogate the interaction between full-length, wild-type HIF-1α and VHL, providing genetic and biochemical evidence that the conserved proline was post-translationally modified to interact with VHL.

Through the use of mass spectrometry, the modification of HIF-1α was found to be the hydroxylation of proline 564. Furthermore, pharmacological inhibitors of prolyl hydroxylation such as cobalt chloride (CoCl2), desferrioxamine (DFO), and

*This investigation was supported by NCI, National Institutes of Health Grants CA67166 (to A. J. G. and N. C. D.) and CA89302 (D. A. C. and P. D. S.). 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.

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1 The abbreviations used are: HRE, hypoxia-responsive element; HIF-1α, hypoxia-inducible factor-1α; VHL, von Hippel-Lindau; DFO, desferrioxamine; DMOG, dimethyloxalyglycine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HKO, HIF-1α knockout; MEF, mouse embryo fibroblast.
dimethylxalylglycine (DMOG) added further support for the importance of the hydroxylation event in regulating HIF-1α under normoxic conditions.

In addition to cobaltous ions and iron chelators, other stresses have been reported to stabilize HIF-1α under normoxic conditions, including transforming oncogenes such as Ras, v-Src, and Akt (12–16). However, it is unclear how oncogenic stresses result in normoxic HIF-1α stabilization. In this study, we generated a specific antibody to the hydroxylated form of proline 564 (Pro-564) to directly determine whether HIF-1α is hydroxylated under normoxic conditions and investigate whether different stresses, including the oncogenes Ras, v-Src, and Akt, stabilize the HIF-1α protein through the inhibition of prolyl hydroxylation.

EXPERIMENTAL PROCEDURES

Reagents—MG132 was purchased from Calbiochem. CoCl2 and DFO were purchased from Sigma. DMOG was a generous gift from Dr. Wayne Zundel.

Antibodies and Immunoblotting—Antisera to hydroxylated HIF-1α were produced in rabbits immunized with a modified peptide of amino acids 558–565 of HIF-1α conjugated to KLH (Custom Antibody Group, Zymed Laboratories Inc.). Hydroxylated HIF-1α antibody was then affinity purified by binding to immobilized modified peptide (SU64, Pierce). Dot blots were performed by serially diluting both modified and unmodified peptide onto nitrocellulose membranes (Bio-Rad). Total peptide levels were visualized by the addition of Coomassie Brilliant Blue R250 (Bio-Rad). For immunoblotting, cells were lysed in radioimmune precipitation assay lysis buffer (1% Nonidet P-40, 0.75% sodium deoxycholate, 0.1% SDS, 150 mm NaCl, 2 mm EDTA, 50 mm sodium fluoride, 10 mm sodium phosphate, pH 7.2) supplemented with 1 mm phenylmethylsulfonyl fluoride, 10 mm sodium orthovanadate, and Complete protease inhibitor cocktail (Roche Molecular Biochemicals). Cells were then scraped and sonicated briefly (10 s). 50 μg of protein (as determined by bicinchoninic acid (BCA) protein assay kit, Pierce) were electrophoresed on 8% SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). Total HIF-1α protein was detected with an anti-HIF-1α antibody from Transduction Laboratories (clone 54). Total HIF-2α protein was detected with an anti-HIF-2α antibody from Novus Biologicals (clone H1667). Anti-GAPDH was from Research Diagnostics, Inc. (clone 6C5).

Cell Lines and Transient Transfections—RCC4 cells, 786-O cells, HIF-1α knockout mouse embryo fibroblasts, and mouse embryo fibroblasts with HIF-1α alleles flanked by loxP sites were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (8, 17, 18). The designation 786 here refers to 786-O. RCC4/VHL and 786/VHL renal carcinoma cell lines with reintroduced VHL were generated by transfecting full-length VHL and selecting with G418. Transient transfections were performed using LipofectAMINE-Plus reagent (Invitrogen) according to the manufacturer’s directions. HIF-1α mutant constructs were generated as described in Ref. 10. Briefly, plasmids were generated using a site-directed mutagenesis kit (QuikChange, Stratagene) and confirmed by DNA sequencing (19).

Hylox—Hyoxia treatment was carried out in a hypoxia chamber (<0.2% O2) (Sheldon Corporation).

Luciferase Assay—Luciferase activity was determined by mixing 10 μl of extracts from 50 × 106 cells and 100 μl of luciferase assay reagent (Promega). Light production was measured for 10 s in a Monolight 2010 Luminesimeter (Analytical Luminescence Laboratory).

RESULTS

Production of a Hydroxy-specific HIF-1α Antibody—To examine the hydroxylation status of the HIF-1α protein under different normoxic stabilizing conditions, we had a polyclonal antibody made against the hydroxylated proline residue 564 of HIF-1α. A synthetic peptide of residues 558–565 of HIF-1α that contained a hydroxylated proline 564 was used to immunize a rabbit for the production of polyclonal antisera. We first probed a serial dilution of modified and unmodified peptides with pre-immune serum, total antisemur, and purified antibody by dot blot. The unpurified antisemur recognized the peptide antigens, whereas the pre-immune serum did not recognize either the hydroxylated or unhydroxylated forms (Fig. 1, compare C with B). Following affinity purification of the hydroxylation-specific antibody from the antiserum, we found that the antibody recognized the hydroxylated form of HIF-1α with at least a 100-fold higher specificity than the unmodified peptide (Fig. 1D). The purified hydroxylation-specific antibody did not, however, recognize peptides made against hydroxylated or unhydroxylated proline 564 (Fig. 1F). Coomassie-stained membranes confirmed the presence of peptide (Fig. 1, A and E). To our knowledge, this is the first antibody that recognizes a specific hydroxylation event.

Hydroxy-specific Antibody Recognizes Normoxic HIF-1α Protein—With the purpose of further examining the specificity of this antibody, we transiently transfected HIF-1α knockout (HKO) mouse embryo fibroblasts (MEFs) and MEFs with HIF-1α alleles flanked by loxP sites (17) with full-length HIF-1α as well as three mutant versions of HIF-1α: proline 402 to alanine (P402A), proline 564 to glycine (P564G), and a double proline 402 and 564 mutant (P402A/P564G) (10). Co-transfection of these mutants and a 5X-HRE-luciferase reporter construct (19) into the MEFs with floxed HIF-1α or HKO MEFs demonstrated an increased ability of these HIF-1α mutants to transactivate (Fig. 2, A and B). Furthermore, by Western blot analysis, these mutants showed increased stability under normoxia (Fig. 2C). We hypothesized that proline 564 when changed to a glycine residue could not be hydroxylated and should not be recognized by a hydroxylation-specific antibody. The proline 402 mutations were used to determine whether the antibody was specific for the hydroxylated form of proline 564 or whether it recognizes other hydroxylated proline residues. Under normoxic conditions, total HIF-1α protein is modestly increased with the proline 402 and 564 alterations, but the hydroxylation-specific antibody recognized only HIF-1α with an intact proline 564 (Fig. 2C). Interestingly, mutation of proline 402 did not affect proline 564 hydroxylation, indicating that hydroxylation of one site does not affect the modification of the other.

To test the hydroxy-specific HIF-1α antibody against the HIF-1α protein under hypoxic conditions, we transiently transfected full-length human HIF-1α into the HKO MEFs. 24 h after transfection, transfected cells were then treated with the proteasome inhibitor MG132 and/or hypoxia for 6 h (Fig. 2D). Because the half-life of the HIF-1α protein under normoxic conditions is very short, estimated to be 5 min (20), and the abundance of HIF-1α protein as a transcription factor is relatively low, we employed the proteasome inhibitor MG132 to increase the abundance of the hydroxylated form of the protein. Western blot analysis of whole-cell extracts showed that hypoxia induced the total HIF-1α protein, and treatment with
MG132 further stabilized HIF-1α. Induction of total HIF-1α by hypoxia is weak because of the already high level of expression of HIF-1α in these cells. The hydroxylated Pro-564 of HIF-1α was only detectable in normoxic cells treated with MG132. Thus, the proteasome inhibitor data support the conclusion that the hypoxia-induced HIF-1α protein is not hydroxylated at proline residue 564.

Hypoxia Mimetics Stabilize Endogenous HIF-1α and HIF-2α by Inhibition of Prolyl Hydroxylation—To determine whether HIF-1α stabilizing factors, under normoxic conditions, inhibit endogenous HIF-1α hydroxylation and, therefore, prevent degradation, RCC4 cells (8) with and without functional VHL were subjected to various HIF-1α stabilizing stresses. Immunoblot analysis of total cell extracts show that RCC4 cells lacking functional VHL had detectable levels of the hydroxylated form of HIF-1α under normoxic conditions, which was further increased with the addition of a proteasome inhibitor (Fig. 3A). In these cells, hydroxylated Pro-564 decreased under hypoxic conditions as well as under treatment with DMOG, CoCl2, and DFO. In contrast, reintroduction of VHL into RCC4 cells (RCC4/VHL) resulted in low levels of hydroxylated HIF-1α under normoxic conditions (Fig. 3B). Hypoxia-treated cells exhibited a substantial decrease in levels of hydroxylated Pro-564 despite a massive increase in total HIF-1α protein. Treatment of cells with a proteasome inhibitor slightly increased the levels of hydroxylated HIF-1α. However, treatment of cells with the


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This is the first example of a hydroxylation-specific antibody. There are numerous antibodies that specifically recognize phosphorylated or acetylated protein motifs. Previously, determination of HIF-1α hydroxylation required recombinant fusion proteins, synthetic peptides, or in vitro transcription/translation (IVTT) and/or mass spectrometry (5–7, 10, 11). These methods require a substantial amount of effort, skill, and, in some cases, specialized equipment. The generation of hydroxylation-specific HIF-1α antibodies will provide an easier and more direct method to investigate how diverse stimuli induce HIF-1α stabilization. In addition, such antibodies will also be useful in the development of diagnostic assays and high throughput screening for prolyl hydroxylase regulatory molecules. Because the immunizing peptide used in this study encompasses a region of functional HIF-1α that is conserved in solid tumors that possess functional VHL. It is noteworthy that hydroxylated HIF-1α Pro-564 was detected in the cells expressing a constitutively active Akt. This finding strongly suggests that a second prolyl hydroxylase-independent mechanism also exists for HIF-1α stabilization under normoxic conditions.

**DISCUSSION**

Prolyl hydroxylase inhibitors DMOG, CoCl₂, and DFO resulted in complete abrogation of Pro-564 hydroxylation. These data indicate that hypoxia and hypoxia mimetics inhibit prolyl hydroxylation, leading to HIF-1α stabilization. Similarly, 786 cells (18), which lack both HIF-1α and VHL, and 786 cells that had wild-type VHL reintroduced (786/VHL) but still lacked HIF-1α were examined to determine the effect of different hypoxia mimetics on HIF-2α. Because the peptide sequence that we employed to generate the antibody is 100% conserved throughout species as well as 90% conserved between HIF-1α and HIF-2α, we hypothesized that the antibody should also recognize the HIF-2α protein. In the 786 cells, which are deficient in both HIF-1α and VHL, we found detectable hydroxylated HIF-2α Pro-531 under all conditions examined (Fig. 3C). Treatment with proteasome inhibitor increased hydroxylated Pro-531, whereas hypoxia, DMOG, CoCl₂, and DFO decreased hydroxylation. In the 786 cells stably expressing wild-type VHL we found hydroxylated HIF-2α in normoxic cells treated with a proteasome inhibitor (Fig. 3D). Similar to what we found with RCC4 cells, hypoxia-treated 786/VHL cells resulted in a substantial increase in total HIF-2α protein without the corresponding increase in hydroxylated HIF-2α. Again, the different hypoxia mimetic stresses, DMOG, CoCl₂, and DFO, resulted in increases in total protein but complete abrogation of the hydroxylated form. Thus, our antibody recognizes hydroxylated forms of HIF-1α and HIF-2α in cells.

**Oncogene Stabilization of HIF-1α Occurs through Two Different Mechanisms—**Ras, v-Src, and Akt have been shown previously to stabilize HIF-1α under normoxic conditions (12, 16, 21). We examined whether these oncogenes increase HIF-1α protein stabilization by inhibition of the hydroxylation of HIF-1α Pro-564. HKO cells were co-transfected with HIF-1α, RasV12, v-Src, or a constitutively active Akt (myristoylated and palmitylated Akt; and a 5X-HRE-luciferase reporter construct (in a 3:1:1 ratio). The introduction of RasV12, v-Src, and Akt had dramatic inductive effects on HIF-1α transactivation ability (Fig. 4A). Conversely, a dominant negative form of Ras, RasN17, had no effect on HIF-1α transactivation. Protein levels of total HIF-1α increased substantially with the cotransfection of RasV12, v-Src, and Akt but not RasN17 (Fig. 4B). Surprisingly, hydroxylated HIF-1α was not detected in the cells transfected with RasV12 or v-Src, even though there was a significant increase in protein levels. This experiment indicated that the oncogenes RasV12 and v-Src increase HIF-1α transactivation potential and stabilize HIF-1α through inhibition of the hydroxylation event. Thus, loss of prolyl hydroxylase activity may be the link between oncogenic activity, HIF-1α, and malignant progression under normoxic conditions. Furthermore, the loss of prolyl hydroxylase activity provides a molecular explanation for the observations that many HIF-1-inducible genes can be observed in solid tumors that possess functional VHL. It is noteworthy that hydroxylated HIF-1α Pro-564 was detected in the cells expressing a constitutively active Akt. This finding strongly suggests that a second prolyl hydroxylase-independent mechanism also exists for HIF-1α stabilization under normoxic conditions.

Genetically and pharmacologically, we were able to demonstrate by Western blot analysis the specificity of an antibody that recognizes only the hydroxylated form of HIF-1α. This antibody solely recognizes hydroxylated proline 564 and is not a pan-hydroxylase antibody as demonstrated by the lack of detection against proline 402 (Figs. 1, D and F and 2C). The mutation of prolines 402 and/or 564 stabilizes the transactivation-competent HIF-1α protein under normoxic conditions (Fig. 2 and Ref. 5). These mutations prevent the amino acid residues from being hydroxylated. In the experiments presented in this paper, only HIF-1α with an intact proline 564 was specifically recognized. The status of hydroxylation of HIF-1α Pro-402 does not appear to affect the hydroxylation of HIF-1α Pro-564 or vice versa, confirming previous reports that the two hydroxylated prolines represent two independent mechanisms of stabilization and transactivation of HIF-1α (10).

Notably, endogenous hydroxylated HIF-1α and HIF-2α proteins were detected under all the conditions examined in the

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*D. A. Chan, unpublished results.*
VHL-deficient renal cell carcinomas RCC4 and 786, respectively, despite oxygen status or treatment with prolyl hydroxylase inhibitors. In contrast, hydroxylated HIF-1α and HIF-2α were detected under normoxia and, to a significantly lesser extent, under hypoxia in the cells with functional VHL. VHL is the E3 ubiquitin ligase responsible for targeting the HIF-1α protein for proteasomal degradation. When VHL is non-functional, the HIF-1α protein is stabilized regardless of oxygen status. These results suggest that when cells shift from normoxia to hypoxia, the hydroxylated protein is degraded and de novo protein synthesis produces HIF-1α or HIF-2α that is not modified. These findings imply that normal protein turnover is the key to eliminating hydroxylated HIF-1α rather than "dehydroxylation" of the protein.

Moreover, DMOG, cobaltous ions, and iron chelators all stabilize a non-hydroxylated form of the HIF-1α protein (5–7). These findings are consistent with previous reports of the effects of cobaltous ions and iron chelators on prolyl hydroxylase activity and indicate that iron is a necessary cofactor in the post-translational modification of HIF-1α. Thus, cobaltous ions, which compete with iron for binding to the prolyl hydroxylase, and iron chelation, which binds available iron, act as prolyl hydroxylase inhibitors and prevent prolyl hydroxylation. These specific prolyl hydroxylase inhibitors appear to completely abolish hydroxylation activity, whereas under hypoxia there still appears to be minimal activity of the hydroxylation in the renal carcinoma cells that have reintroduced VHL.

An examination of HIF-1α protein in RCC4 and HIF-2α in 786 cells indicates that there may be an additional pathway that targets the α-subunit of HIF to the proteasome, because an increase in HIF-α protein levels occurs with the addition of a proteasome inhibitor. If VHL is the sole mechanism of targeting HIF-1α or HIF-2α protein to the proteasome, then blocking the proteasome should not increase total HIF-1α or HIF-2α protein levels. Taken together with the observation that there is a low level of hypoxic induction of HIF-1α in RCC4 and HIF-2α in 786 cells, we conclude that a secondary mechanism for HIF-1α and HIF-2α degradation independent of VHL also exists. It is not yet clear what regulates this mechanism, although a recent report suggests that Hsp90 is involved in a VHL-independent HIF-1α degradative pathway (22). A molecular chaperone protein, Hsp90, plays a key role in the conformation and function of its numerous client proteins. The data presented above suggest that the Akt oncogene, a known substrate of Hsp90 (23), may impinge on this means of regulation.

We have shown that normoxic induction of HIF-1α by the oncogenes RasV12 and v-Src appears to be mediated by inhibition of the prolyl hydroxylation event, whereas Akt stabilization appears to be independent of the prolyl hydroxylation event. The above data suggest that Akt functions through a different mechanism than RasV12 and v-Src. Total HIF-1α increases similarly, although there is no apparent hydroxylated HIF-1α following the overexpression of RasV12 or v-Src. It is possible that HIF-1α stabilization by Akt overexpression is
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not a prolyl hydroxylation-independent mechanism but that the increase in HIF-1α is due to increased protein translation or decreased protein degradation. More specifically, Akt overexpression could result in increased transcription or translation of HIF-1α, saturating VHL or other degradation machinery. Akt could also impede the degradation of HIF-1α by Akt compared with RasV12 or v-Src may arise from differential regulation of the two crucial proline (Pro-402 and Pro-564) sites in the oxygen degradation domain.

More intriguing is the prospect that normoxic induction of stabilization of HIF-1α through inhibition of the VHL complex either directly or indirectly. More intriguing is the prospect that normoxic induction of HIF-1α by Akt compared with RasV12 or v-Src may arise from differential regulation of the two crucial proline (Pro-402 and Pro-564) sites in the oxygen degradation domain.

Interestingly, Ras and v-Src both function through the same pathway in this case, which suggests that a wide variety of disparate normoxic stabilizing stresses may induce HIF-1α through a common mechanism. Normoxic expression of HIF-1α may act as a protumorigenic factor, even through a common means. Normoxic expression of HIF-1α may provide a therapeutic advantage.

Acknowledgments—We thank Dr. Shing-Erh Yen and Glenn Ruiz at Zymed Laboratories Inc for excellent technical assistance and helpful discussion, Drs. Esther M. Hammond and Dawn L. Zink for immunohistochemical analyses, and Dr. Scott M. Welford for constructive criticism. We also thank Eric Alemany and Sharon Clarke for all their technical support. We are also grateful to Trent Watkins for insightful deliberations and infinite patience.

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