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12 March 2001; accepted 16 March 2001
Published online 22 March 2001;
10.1126/science.1060622
Include this information when citing this paper.

HIFα Targeted for VHL-Mediated Destruction by Proline Hydroxylation: Implications for O_2 Sensing

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HIF (hypoxia-inducible factor) is a transcription factor that plays a pivotal role in cellular adaptation to changes in oxygen availability. In the presence of oxygen, HIF is targeted for destruction by an E3 ubiquitin ligase containing the von Hippel–Lindau tumor suppressor protein (pVHL). We found that human pVHL binds to a short HIF-derived peptide when a conserved proline residue at the core of this peptide is hydroxylated. Because proline hydroxylation requires molecular oxygen and Fe^{2+}, this protein modification may play a key role in mammalian oxygen sensing.

How cells sense changes in ambient oxygen is a central question in biology. In mammalian cells, lack of oxygen, or hypoxia, leads to the stabilization of a sequence-specific DNA binding transcription factor called HIF, which transcriptionally activates a variety of genes linked to processes such as angiogenesis and glucose metabolism (*1–4*). HIF binds to DNA as a heterodimer consisting of an α subunit and a β subunit.

Von Hippel–Lindau (VHL) disease is a hereditary cancer syndrome characterized by the development of highly vascular tumors that overproduce hypoxia-inducible mRNAs such as vascular endothelial growth factor (VEGF) (*5*). The product of the VHL tumor suppressor gene, pVHL, is a component of a multiprotein complex that bears structural and functional similarity to SCF (Skp1/Cdc53 or Cullin/F-box) ubiquitin ligases (*6–11*). In the presence of oxygen, pVHL, in association with elongin B and elongin C, binds directly to HIFα subunits and targets them for polyubiquitination and destruction (*7–10*). Cells lacking functional pVHL cannot degrade HIF and thus overproduce mRNAs encoded by HIF target genes (*12*). Here, we investigate the mechanism by which hypoxia prevents the destruction of HIF.

VHL interacts with a modified form of HIF. We first followed up on earlier observations that cobalt chloride or iron chelators such as desferrioxamine (i) stabilize HIF and lead to transcriptional activation of its target genes (*1–4*), (ii) inhibit the binding of pVHL to HIF (*12*), and (iii) inhibit HIF polyubiquitination by pVHL in vitro (*7*). To study this further, we treated pVHL-defective renal carcinoma cells with increasing amounts of cobalt chloride or desferrioxamine. As shown earlier, the untreated cells contained high levels of HIF-2α, which bound directly to recombinant pVHL–elongin B–elongin C (VBC) (*7, 12, 13*) (Fig. 1A). In contrast, VBC did not recognize HIF-2α isolated from cells treated with cobalt chloride or desferrioxamine.

A recent study indicated that hypoxia, in contrast to cobalt chloride and desferrioxamine, inhibits HIF polyubiquitination but not the physical association of pVHL and HIF (*12*). This suggests that physiological regulation of HIF by hypoxia is mechanistically distinct from the pharmacological effects of cobalt chloride and desferrioxamine. Exposure of cell extracts to oxygen in this earlier study, however, might have allowed for reformation of pVHL-HIF complexes after lysis. To explore these observations further, we grew mouse cells (ts20) with a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme (*14*) at the nonpermissive temperature under hypoxic or normoxic conditions so that HIF would accumulate in the presence or absence of oxygen (*15*). Although comparable levels of HIF accumulated in these two settings, VBC only recognized the HIF from normoxic cells (Fig. 1B). Thus, the interaction of pVHL with HIF appears to be governed by a posttranslational modification of HIF that is oxygen- and iron-dependent.

pVHL binds to a region of HIF-1α called the oxygen-dependent degradation domain (ODD) (*7*). We had observed that pVHL bound to HIF produced in rabbit reticulocyte lysate but did not bind to HIF produced in wheat germ extracts or in *Escherichia coli* (*16*). Furthermore, wheat germ– or *E. coli*–derived HIF acquired pVHL binding activity after preincubation with human, rabbit, or *Xenopus* cell extracts at 37°C (*16*). For example, glutathione *S*-transferase (GST)–ODD fusion proteins produced in *E. coli* were not recognized by VBC unless preincubated with a rabbit reticulocyte lysate (Fig. 1C). VBC did not recognize GST-ODD fusion proteins incubated with a heat-inactivated reticulocyte lysate (Fig. 1D). These results indicate that pVHL recognizes a modified form of HIF and that this modification is carried out by a factor present in a variety of vertebrate cell extracts.

To determine the nature of this modification, we first narrowed the region of HIF that binds to pVHL. Gal4-HIF fusion proteins containing HIF residues 555 to 575 bound specifically to immobilized GST-VBC complexes (Fig. 2, A and E) (*13, 17*). Likewise, a biotinylated peptide corresponding to HIF residues 556 to 575 [henceforth HIF(556-575)] bound to pVHL after preincubation with reticulocyte lysate (Fig. 2B) (*18*). As noted by others, this region of HIF contains a highly conserved collinear sequence, Met-Leu-Ala-Pro-Tyr-Ile-Pro-Met (Fig. 2E), which, when mutated to eight consecutive alanines, leads to HIF stabilization (*19*). An alanine scan of this region showed that Leu^{562} and Pro^{564} were essential for specific binding to pVHL (Fig. 2B). In contrast, mutation of the one potential phosphoacceptor in this peptide, Tyr^{565}, did not affect pVHL binding, consistent with an earlier study in which a $Tyr^{565} \rightarrow$ Phe mutation did not affect HIF stability (*20*). Moreover, phosphatase treatment did not affect the binding of pVHL to GST-ODD in these assays (*16*).

Mutation of either Leu^{562} or Pro^{564} to Ala in the context of full-length HIF-1α or a Gal4-ODD fusion protein abrogated pVHL

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binding activity (Fig. 2, C and D). We noted that Gal4-ODD synthesized in reticulocyte lysate contained an electrophoretically distinct band that was absent from wheat germ–derived Gal4-ODD preparations (Fig. 2D). The corresponding protein bound to VBC and was undetectable among the Leu^{562} → Ala (L562A) and Pro^{564} → Ala (P564A) translation products, suggesting that it might contain a posttranslational modification of Leu^{562} or Pro^{564}. Two-dimensional (2D) protein gel electrophoresis suggested that this putative modification did not involve a change in protein charge (*16*).

Consistent with the pVHL binding assays, Gal4-HIF fusion proteins with the L562A or P564A mutations displayed diminished pVHL-dependent polyubiquitination in vitro (Fig. 3A) (*13*). Qualitatively similar results were obtained with the corresponding full-length HIF-1α species (*16*). Likewise, full-length HIF-1α P564A and HIF-1α L562A were more stable than wild-type HIF-1α in degradation assays performed with *Xenopus* extracts (*21*) (Fig. 3B).

Identification of the HIF modification as proline hydroxylation. We suspected that the HIF modification might be proline hydroxylation because prolyl hydroxylases require oxygen and iron (*22*). To test this, we incubated biotinylated HIF(556-575) peptides with rabbit reticulocyte lysate, eluted the peptides with free biotin, and analyzed them by mass spectrometry (*23*). In these experiments we replaced Met^{561} and Met^{568} with alanine to prevent spurious oxidation of the methionines. These substitutions did not affect pVHL binding (*16*). The HIF peptide samples that had been pretreated with rabbit reticulocyte lysate contained a second peak in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis that corresponded to an increase in molecular weight of 16 (Fig. 4A). This peak was not

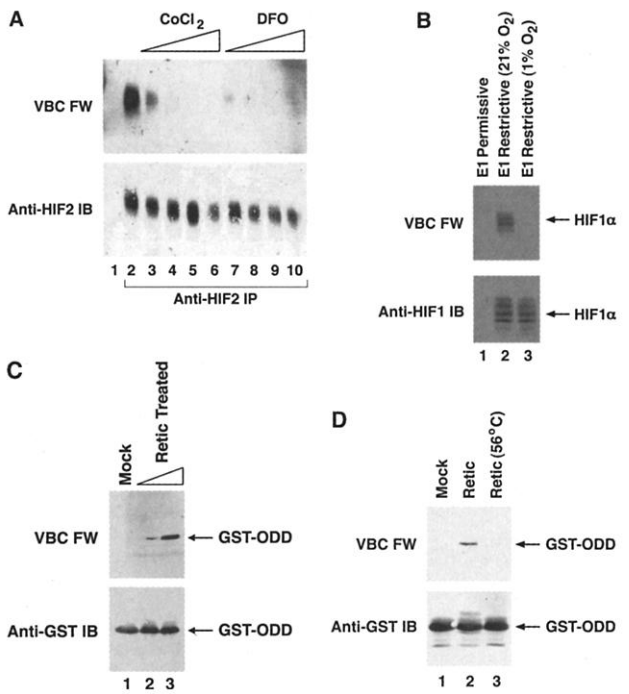

Fig. 1. pVHL binds to a modified form of HIF. (**A**) pVHL-defective renal carcinoma cells were treated with increasing amounts of desferrioxamine (DFO) (2, 10, 100, or 1000 μM; lanes 7 to 10) or cobalt chloride (2, 10, 100, or 1000 μM; lanes 3 to 6) and immunoprecipitated with anti-Cul2 (control) (lane 1) or anti–HIF-2α (lanes 2 to 10). Bound proteins were detected by anti–HIF-2α immunoblot (IB) or by far-western (FW) analysis with purified VBC complexes. (**B**) VBC farwestern and anti–HIF-1α immunoblot analysis of ts20 cells grown at the restrictive temperature under hypoxic or normoxic conditions. (**C** and **D**) About 1 μg of GST–HIF-1α(530-652), containing the oxygen-dependent degradation domain (ODD), was produced in *E. coli*, recovered on glutathione-Sepharose, and incubated with 50 μl of rabbit reticulocyte lysate for 90 min at 30°C. In lane 3 of (D), the reticulocyte lysate was first heat-inactivated for 20 min. After stringent washes, the GST-ODD protein was subjected to VBC farwestern and anti-GST immunoblot analysis.

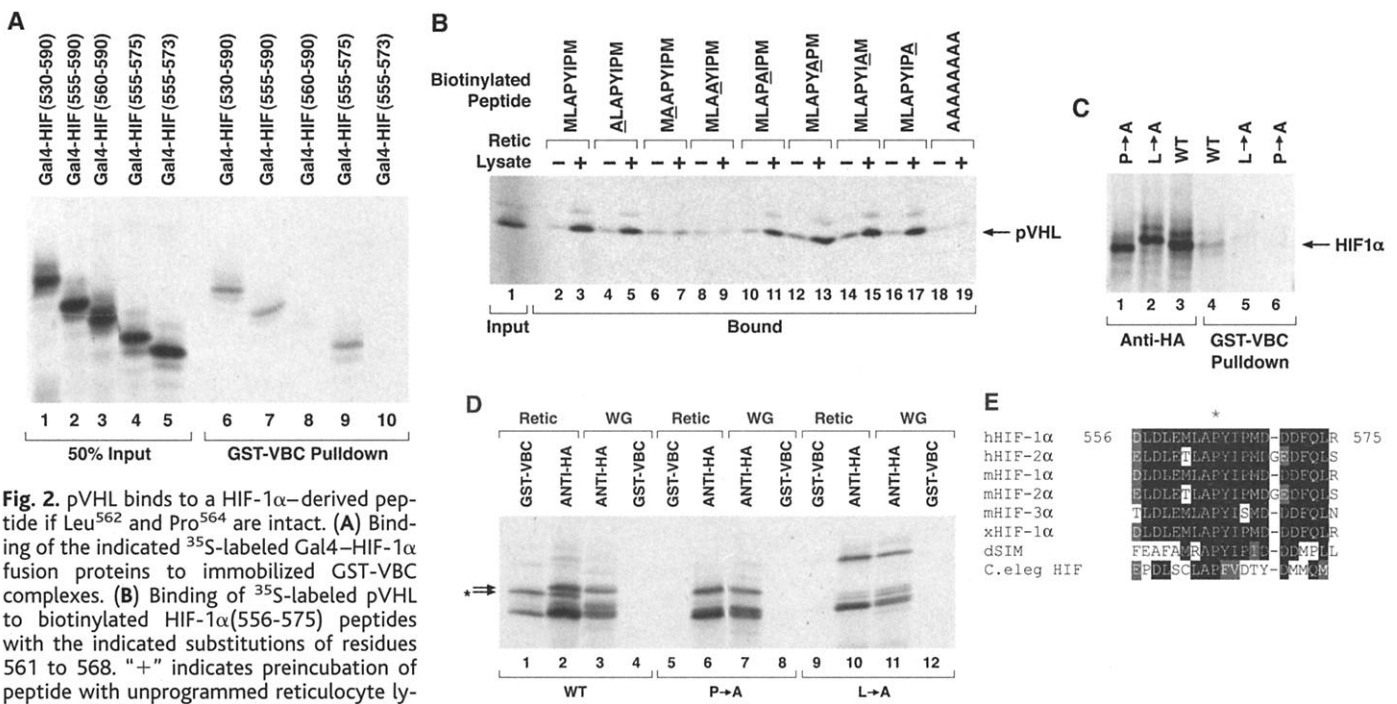

Fig. 2. pVHL binds to a HIF-1α–derived peptide if Leu^{562} and Pro^{564} are intact. (**A**) Binding of the indicated ^{35}S-labeled Gal4–HIF-1α fusion proteins to immobilized GST-VBC complexes. (**B**) Binding of ^{35}S-labeled pVHL to biotinylated HIF-1α(556-575) peptides with the indicated substitutions of residues 561 to 568. "+" indicates preincubation of peptide with unprogrammed reticulocyte lysate before addition of pVHL. (**C** and **D**) ^{35}S-labeled wild-type (WT), P564A, and L562A full-length HA–HIF-1α (C) and Gal4-HA–HIF-1α(530-652) (D) proteins were immunoprecipitated with anti-HA or captured with immobilized GST-VBC complexes. WG, wheat germ extract; Retic, rabbit reticulocyte lysate. The reason for the anomalous migration of the L562A mutant in (D) is not known. (**E**) Conservation of Leu^{562} and Pro^{564} among human (h),mouse (m), *Xenopus* (x), *Drosophila* (d), and *Caenorhabditis elegans* HIF orthologs and paralogs. Single-letter abbreviations for amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

detectable in peptide preparations before incubation with reticulocyte lysate, nor in reticulocyte-treated L562A and P564A peptides (Fig. 4A) (*16*). Electrospray ion trap tandem mass spectrometry (MS/MS) confirmed the addition of +16 at Pro^{564} and excluded such a modification of Leu^{562} (*24*). Moreover, ion trap MS/MS of the reticulocyte-treated HIF(556-575) peptide produced a product ion spectrum that was identical to that obtained with a synthetic HIF(556-575) peptide containing hydroxyproline at position 564 (*16*). We translated Gal4-HIF(555-575) in the presence of [^{3}H]proline using rabbit reticulocyte lysate or wheat germ extract,

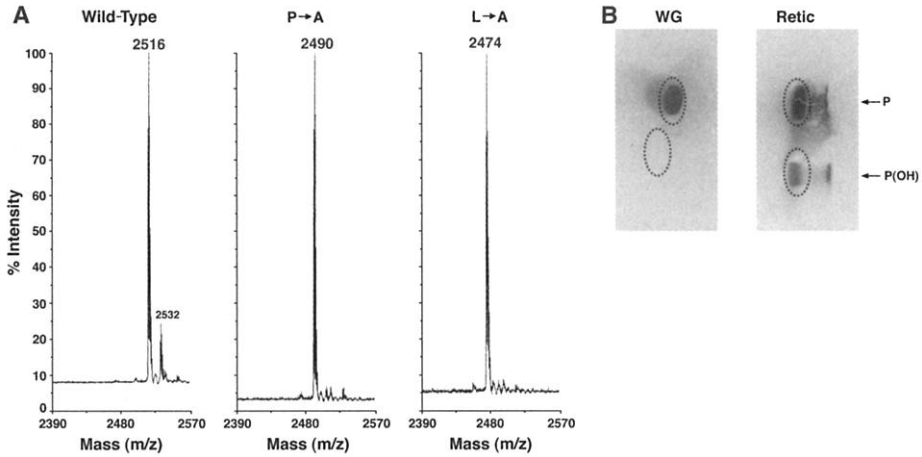

Fig. 4. Proline hydroxylation linked to pVHL binding. (**A**) MALDI-TOF analysis of wild-type, P564A, and L562A biotinylated HIF(556-575) peptides after incubation with rabbit reticulocyte lysate. (**B**) Gal4-HA–HIF(555-575) was translated in vitro in the presence of [^{3}H]proline with rabbit reticulocyte lysate or wheat germ extract, hydrolyzed, and analyzed by TLC. Dashed circles indicate positions of ninhydrin-stained proline and hydroxyproline markers.

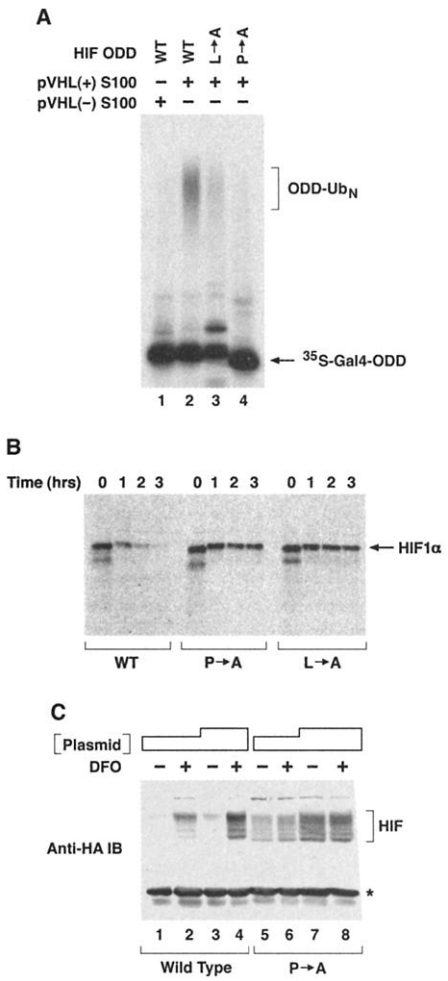

Fig. 3. Ubiquitination and degradation of HIF linked to Leu^{562} and Pro^{564}. (**A**) In vitro ubiquitination of ^{35}S-labeled wild-type, L562A, and P564A Gal4-HA–HIF-1α(530-652) (HIF-ODD) in the presence of S100 extracts prepared from pVHL-defective renal carcinoma cells stably transfected with a vector producing wild-type pVHL or with empty vector. (**B**) In vitro degradation of ^{35}S-labeled wild-type, L562A, and P564A HIF-1α in *Xenopus* egg extracts. (**C**) Anti-HA immunoblot analysis of COS7 cells transiently transfected with 1.5 μg (lanes 1, 2, 5, and 6) or 3.5 μg (lanes 3, 4, 7, and 8) of plasmids encoding wild-type or P564A HA–HIF-1α and then transferred to media that did or did not contain desferrioxamine.

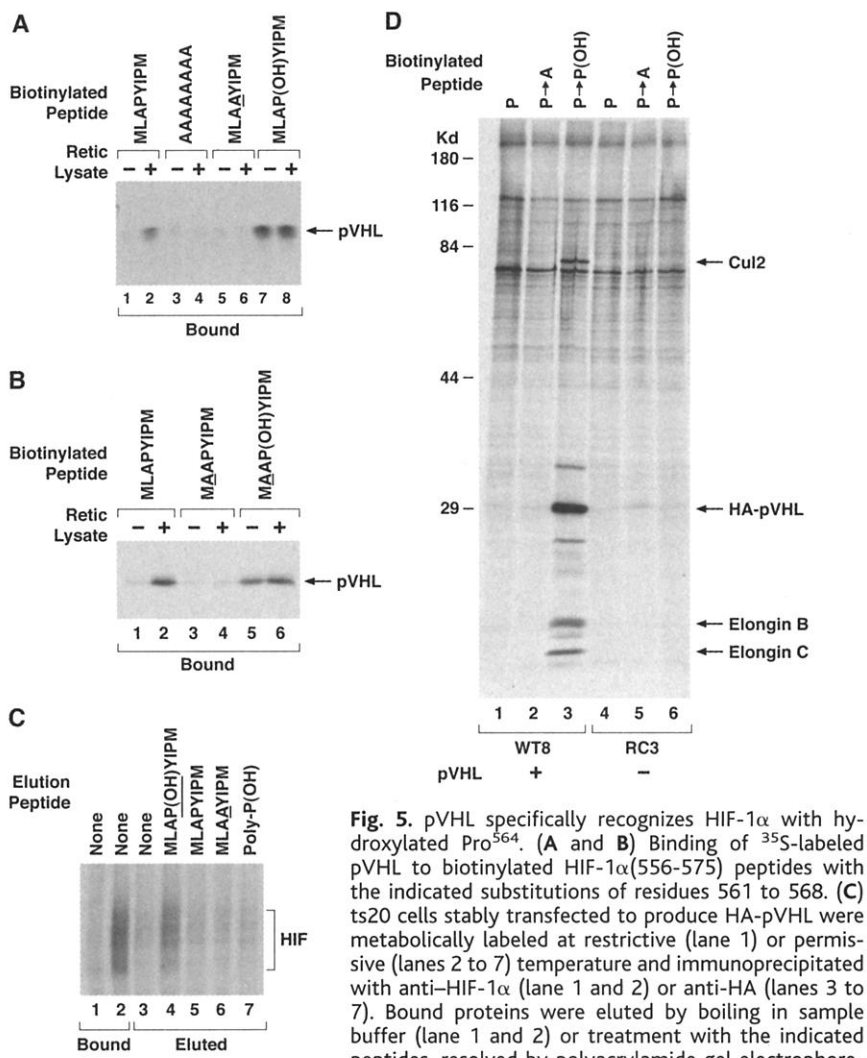

Fig. 5. pVHL specifically recognizes HIF-1α with hydroxylated Pro^{564}. (**A** and **B**) Binding of ^{35}S-labeled pVHL to biotinylated HIF-1α(556-575) peptides with the indicated substitutions of residues 561 to 568. (**C**) ts20 cells stably transfected to produce HA-pVHL were metabolically labeled at restrictive (lane 1) or permissive (lanes 2 to 7) temperature and immunoprecipitated with anti–HIF-1α (lane 1 and 2) or anti-HA (lanes 3 to 7). Bound proteins were eluted by boiling in sample buffer (lane 1 and 2) or treatment with the indicated peptides, resolved by polyacrylamide gel electrophoresis, and detected by autoradiography. (**D**) pVHL-defective renal carcinoma cells that had been stably transfected with a vector producing wild-type pVHL (WT8) or with empty vector (RC3) were metabolically labeled with [^{35}S]methionine, lysed, and incubated with immobilized biotinylated HIF-1α(556-575) peptides with the indicated substitutions of residue 564. Specifically bound proteins were detected by autoradiography.

isolated it using an electrophoretic gel, and subjected it to acid hydrolysis and thin-layer chromatography (TLC) (*17, 25*). The Gal4-HIF produced in rabbit reticulocyte lysate, but not in wheat germ, contained hydroxyproline (Fig. 4B).

As expected, the HIF(556-575) peptide containing hydroxyproline at position 564 bound to pVHL with or without pretreatment with reticulocyte lysate (Fig. 5A). The mass spectrometry analysis of the L562A peptide showed that Leu^{562} was required for HIF modification (Fig. 4A), but left open the possibility that it was not required for binding to pVHL. Indeed, pVHL bound to a HIF(556-575) peptide with the L562A substitution and hydroxyproline at residue 564 (Fig. 5B). This suggests that Leu^{562} facilitates hydroxylation of Pro^{564}.

To explore whether the hydroxylated HIF peptide interacts with cell-derived pVHL complexes, we used two approaches. First, we engineered ts20 cells to produce hemagglutinin (HA)-tagged pVHL (*26*). We found that HIF bound to HA-pVHL at the restrictive temperature could be eluted by the hydroxylated HIF(556-575) peptide but not by the unmodified peptide (*27*) (Fig. 5C). Moreover, HIF was not eluted by the HIF(556-575) P564A peptide or by a polyhydroxyproline peptide (Fig. 5C). Second, we carried out affinity chromatography with immobilized peptides and metabolically labeled matched renal carcinoma cells that do (WT8) or do not (RC3) produce HA-pVHL (*18, 27, 28*). The hydroxylated HIF(556-575) peptide specifically bound to pVHL as well as to proteins with the expected electrophoretic mobilities of the pVHL-associated proteins elongin B, elongin C, and Cul2 (Fig. 5D). These results indicate that pVHL recognizes a proline-hydroxylated epitope present in HIF-1α. Consistent with this idea, a HIF-1α mutant containing a P564A mutation showed enhanced stability in COS7 cells and was insensitive to the hypoxia-mimetic desferrioxamine (Fig. 3C).

Discussion. Our findings extend the similarities between SCF complexes and VBC complexes. Like an F-box protein, the polyubiquitination function of pVHL is linked to its ability to bind to a posttranslationally modified determinant on its target. F-box proteins recognize specific phosphopeptides, whereas pVHL recognizes a proline-hydroxylated peptide (*11*). It will be important to determine whether pVHL has other polyubiquitination targets and, if so, whether they are similarly regulated by proline hydroxylation.

The results of our heat inactivation studies, coupled with our findings on the specific hydroxylation of Pro^{564} rather than Pro^{567} and the influence of Leu^{562}, suggest that HIF hydroxylation is carried out by an enzyme. The same conclusion is reached by Jaakkola *et al.* (*29*). The vertebrate type I and type II prolyl 4-hydroxylases are tetramers consisting of two α subunits [α(I) and α(II), respectively] and two common β subunits (*22*). These enzymes require Fe^{2+}, molecular oxygen, 2-oxoglutarate, and ascorbate and act upon collagen and other proteins that contain collagen-like sequences. The pVHL binding peptide present in HIF does not closely resemble the naturally occurring or synthetic prolyl hydroxylation targets identified to date. Moreover, HIF is intracellular, whereas the majority of proline hydroxylase activity is associated with the endoplasmic reticulum where it is required for normal collagen biosynthesis. It is therefore unlikely that HIF is modified by the type I and type II prolyl 4-hydoxylases.

The requirement of molecular oxygen and iron for proline hydroxylase activity would potentially explain the stabilization of HIF observed under hypoxic conditions or after treatment with agents that eliminate or compete with iron. This notion, however, may be oversimplified. First, the prolyl hydroxylases that modify collagen are active in hypoxic cells (*30, 31*). Second, earlier studies indicated that oxygen sensing involved changes in reactive oxygen species, protein phosphorylation, and protein nitrosylation (*1–4*). Conceivably, these changes directly or indirectly affect the hydroxylation of HIF. Finally, our findings do not provide an immediate explanation for the observation that hypoxic induction of HIF is prevented in the presence of heme inhibitors such as carbon monoxide (*1–4*).

Tissue ischemia is a major cause of morbidity and mortality. In principle, drugs that stabilize HIF may augment angiogenesis and the adaptation to chronic hypoxia. Several small-molecule proline hydroxylase inhibitors have been developed as antifibrotic agents and can now be tested in ischemia models (*22, 32–34*).

The activation of HIF by hypoxia is complex and involves changes in protein stability, nuclear localization, DNA binding capability, and transcriptional activation function (*1–4*). The knowledge that proline hydroxylation governs HIF turnover in the presence of oxygen should facilitate dissection of the mechanisms underlying these various aspects of HIF regulation. Moreover, one can now ask whether proline hydroxylation is a general feature of the cellular response to changes in oxygen, and whether this modification is used in other signaling pathways.

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17. Coupled in vitro transcription-translation of ^{35}S-labeled proteins was performed according to the manufacturer's instructions (TNT; Promega, Madison, WI). In vitro translation of ^{3}H-P-labeled Gal4-HIF(555-575) was done similarly in a 2-ml reaction containing 450 μl of L-[2,3,4,5-^{3}H]proline (New England Nuclear).
18. For peptide binding studies, 1 μg of biotinylated peptide was bound to 30 μl of monomeric avidin agarose (Pierce). Where indicated, the peptide was preincubated with 50 μl of rabbit reticulocyte lysate for 90 min at 30°C. The agarose was then washed three times with NETN [20 mM tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) and used in binding reactions containing 4 μl of ^{35}S-labeled HA-pVHL in 500 μl of EBC or 500 μl of ^{35}S-labeled cell extract (equivalent to cells from a subconfluent 100-mm dish). After a 1-hour incubation at 4°C, the agarose was washed four times with NETN. Bound proteins were eluted by boiling in sample buffer containing SDS and detected by autoradiography.
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an isolation width of 2.5 daltons and relative collision energy of 30%.
24. See *Science* Online (www.sciencemag.org/cgi/content/full/1059817/DC1).
25. We immunoprecipitated 2 ml of ^{3}H-P-labeled Gal4-HIF(555-575) in vitro translated with 50 μg of antibody to HA (anti-HA; 12CA5, Roche); the product was resolved on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Gal4-HIF(555-575) was visualized by autoradiography, and the corresponding region of the membrane was excised and hydrolyzed by incubation in 100 μl of 10 N HCl at 105°C for 3 hours. Samples were evaporated to dryness, resuspended in 20 μl of H_2O containing 10 μg of unlabeled L-proline and trans-4-hydroxy-L-proline (Sigma) (as standards), and resolved by 2D TLC using phenol-distilled H_2O in the first dimension and *N*-butanol–acetic acid–H_2O in the second dimension [J. Ludlow, R. Consigli, *J. Virol.* **63**, 2881 (1989)]. After visualization of standards with ninhydrin, radiolabeled proline was detected by autoradiography.
26. ts20 cells were transfected with pIRES-HA-VHL, pIRES-HA-VHL (Y98H), or pIRES-Neo (Invitrogen) and selected in the presence of G418 (1 mg/ml). Individual G418-resistant colonies were isolated using cloning cylinders and expanded. Cells producing HA-VHL or HA-VHL (Y98H) were identified by anti-HA immunoblot analysis.
27. ts20 cells were grown at restrictive or permissive temperature for 14 hours, methionine-starved for 90 min, and then grown in methionine-free media supplemented with [^{35}S]methionine (500 μCi/ml) for 90 min. Cells were washed once with cold PBS, lysed in EBC, and immunoprecipitated with anti-HA (12CA5, Roche) or anti–HIF-1α (NB100-105, Novus). After five washes with NETN, bound proteins were eluted by boiling in sample buffer or by incubation in 65 μl of PBS containing 7 μg of the indicated peptide. 786-O subclones were starved for 1 hour, grown in methionine-free media supplemented with [^{35}S]methionine (500 μCi/ml) for 3 hours, washed once with ice-cold PBS, and lysed in EBC.
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35. We thank J. Lee and P. Morrison for carrying out the MALDI-TOF analysis, H. Ozer for the ts20 cells, M. Kirschner for help with the *Xenopus* degradation assays, J. Crawford for expert peptide synthesis, M. Modabber for help with artwork, and C. W. Park for contributions to the early stages of the work. Supported by grants from NIH and the Murray Foundation.

12 February 2001; accepted 19 March 2001
Published online 5 April 2001;
10.1126/science.1059817
Include this information when citing this paper.

Targeting of HIF-α to the von Hippel–Lindau Ubiquitylation Complex by O_2-Regulated Prolyl Hydroxylation

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Hypoxia-inducible factor (HIF) is a transcriptional complex that plays a central role in the regulation of gene expression by oxygen. In oxygenated and iron replete cells, HIF-α subunits are rapidly destroyed by a mechanism that involves ubiquitylation by the von Hippel–Lindau tumor suppressor (pVHL) E3 ligase complex. This process is suppressed by hypoxia and iron chelation, allowing transcriptional activation. Here we show that the interaction between human pVHL and a specific domain of the HIF-1α subunit is regulated through hydroxylation of a proline residue (HIF-1α P564) by an enzyme we have termed HIF-α prolyl-hydroxylase (HIF-PH). An absolute requirement for dioxygen as a cosubstrate and iron as cofactor suggests that HIF-PH functions directly as a cellular oxygen sensor.

HIF is a key regulator of responses to hypoxia, occupying a central position in oxygen homeostasis in a wide range of organisms (*1*). Among its transcriptional targets are genes with critical roles in angiogenesis, erythropoiesis, energy metabolism, vasomotor function, and apoptotic/proliferative responses. HIF is essential for normal development (*2*) and plays a key role in pathophysiological responses to ischemia/hypoxia as well as in tumor growth and angiogenesis (*1*). The HIF DNA binding complex is a heterodimer of α and β subunits (*3*). In oxygenated cells, the α subunits are unstable, being targeted for proteosomal destruction by specific degradation domains (*4–7*). This process is dependent on the von Hippel–Lindau tumor suppressor (pVHL) (*8*), which serves as the recognition component of a ubiquitin ligase (*9*, *10*) that promotes ubiquitin-dependent proteolysis of HIF-α (*11–14*). In hypoxic cells, HIF-α degradation is suppressed, leading to transcriptional activation of target genes. Here we aimed to define the mechanisms that regulate HIF-α degradation by oxygen.

O_2-regulated association between HIF-α and pVHL. Classical findings that cobaltous ions and iron chelators mimic the action of hypoxia on the HIF system have led to the suggestion that a specific ferroprotein oxygen sensor might underlie the process (*15–17*). In accordance with this, we previously found that treatment of cells with cobaltous ions and iron chelators prevented the HIF-α/pVHL association, suggesting that the oxygen sensing mechanism might impinge directly on this protein interaction (*8*). Surprisingly, these studies indicated that the HIF-α/pVHL complex could be retrieved intact from hypoxic cells. Given the rapidity of pVHL-dependent proteolysis of HIF-α in oxygenated cells, we postulated that reoxygenation of cell extracts during cell lysis might promote the HIF-α/pVHL interaction in vitro. To test this, we repeated the pVHL co-immunoprecipitation experiments using extracts of ^{35}S-methionine/cysteine-labeled cells that had been exposed to hypoxia and (i) harvested in a hypoxia workstation using deoxygenated buffers (*18*) or (ii) harvested conventionally. These experiments were performed with stably transfected renal carcinoma cells expressing hemagglutinin (HA)-tagged VHL (RCC4/VHL.HA) (*11*). As reported previously (*8*), immunoprecipitation with antibody to HA captured HIF-α subunits (HIF-1α and HIF-2α) efficiently from proteasomally blocked normoxic cells. However, in cells that had been exposed to hypoxia and extracted in deoxygenated buffers, capture of HIF-α was strikingly reduced (Fig. 1A, compare lanes 2 and 4). This contrasted with the result obtained with conventional buffers, where HIF-α subunits were captured very efficient-

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