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.
28. O. Iliopoulos, A. Kibel, S. Gray, W. G. Kaelin, *Nature Med.* **1**, 822 (1995).
29. P. Jaakkola *et al.*, *Science* **292**, 468 (2001).
30. Y. Takahashi, S. Takahashi, Y. Shiga, T. Yoshimi, T. Miura, *J. Biol. Chem.* **275**, 14139 (2000).
31. C. Levene, C. Bates, *Biochim. Biophys. Acta* **444**, 446 (1976).
32. M. Bickel *et al.*, *Hepatology* **28**, 404 (1998).
33. T. Franklin, W. Morris, P. Edwards, M. Large, R. Stephenson, *Biochem. J.* **353**, 333 (2001).
34. L. Friedman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4736 (2000).
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Targeting of HIF-α to the von Hippel–Lindau Ubiquitylation Complex by O_2-Regulated Prolyl Hydroxylation

Panu Jaakkola,[1]* David R. Mole,[1]* Ya-Min Tian,[1] Michael I. Wilson,[1] Janine Gielbert,[2] Simon J. Gaskell,[2] Alexander von Kriegsheim,[3] Holger F. Hebestreit,[3] Mridul Mukherji,[4] Christopher J. Schofield,[4] Patrick H. Maxwell,[1]†‡ Christopher W. Pugh,[1]†‡ Peter J. Ratcliffe[1]†‡

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-

[1]The Henry Wellcome Building of Genomic Medicine, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, UK. [2]Michael Barber Centre for Mass Spectrometry, Department of Chemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, UK. [3]Glycobiology Institute, University of Oxford, South Parks Road, Oxford OX1 3QU, UK. [4]The Oxford Centre for Molecular Sciences and The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, UK.

*These authors contributed equally to the work.
†The contribution of the three senior authors was equivalent.
‡To whom correspondence should be addressed. E-mail: peter.ratcliffe@imm.ox.ac.uk, cwpugh@enterprise.molbiol.ox.ac.uk, pmaxwell@hammer.imm.ox.ac.uk

ly (Fig. 1A, lane 3). These results indicate that the classical features of regulation by oxygen and iron availability are reflected in the HIF-α/pVHL interaction in vivo and that promotion of the interaction mediated by oxygen can occur rapidly during the preparation of a cell extract.

To investigate regulation of the HIF-α/pVHL interaction in vitro, we produced ^{35}S-methionine–labeled HIF-1α subunits and pVHL.HA separately by in vitro transcription translation (IVTT) in reticulocyte lysates under different conditions, mixed them, and assayed them for interaction (*19*). Supplementary Fe(II) (ferrous chloride, 100 μM) in the HIF-1α IVTT greatly enhanced capture by pVHL.HA, whereas addition of Co(II) (cobaltous chloride, 100 μM) or desferrioxamine (DFO, 100 μM) to the HIF-1α IVTT greatly diminished capture (Fig. 1, B and C). In contrast, pVHL IVTTs performed under these conditions were all equally effective in supporting the HIF-1α interaction. Furthermore, HIF-1α produced in the hypoxic workstation had a reduced ability to interact with pVHL (Fig. 1D). Using reticulocyte IVTTs of Gal4/HIF-1α/VP16 fusion proteins, we found that the regulated interaction with pVHL required only HIF-1α residues 556 to 574 (Fig. 1E). We then surveyed the interaction potential of a series of recombinant pVHL and HIF-1α products generated in various prokaryotic and eukaryotic expression systems (*20*). Both mammalian and bacterially expressed pVHL interacted with mammalian HIF-1α. However, only HIF-1α derived from mammalian expression systems interacted with pVHL. Taken together, the findings indicate that a factor in mammalian cells was necessary for the interaction and that this factor operated in an iron- and oxygen-dependent manner.

Enzymatic modification of HIF-1α. We immunopurified a Gal/HIF-1α/VP16 fusion protein expressing HIF-1α residues 549 to 582 from IVTT reactions prepared in the presence of DFO, using anti-Gal. After various treatments, the fusion protein was tested for its ability to bind ^{35}S-methionine–labeled pVHL (*21*). Treatment with Fe(II) alone in the presence of oxygen did not promote interaction, whereas treatment with cell extract promoted interaction in a manner that was iron- and oxygen-dependent (Fig. 2A). Human HIF-1α produced in insect cells required pretreatment with mammalian extract for interaction with pVHL (*22*) (Fig. 2B). The interaction-promoting activity was markedly reduced by cooling, was inactivated by preheating to 60°C for 10 min (Fig. 2C), and did not pass through a 5-kD ultrafilter. Titration of Fe(II) supplementation indicated full activation at 5 μM (*23*). Treatment of the cell extracts with 1 M NaCl abrogated the interaction-promoting activity, whereas similar treatment of the HIF fusion protein after modification by cell extract did not reduce its ability to capture pVHL (Fig. 2D). Likewise, treatment of the HIF fusion protein with phosphatase or DFO after modification by cell extract did not prevent pVHL capture. These results suggested that HIF-1α is a substrate for an enzyme-mediated modification that is not phosphorylation. Further analysis indicated that the enzyme activity was unaffected by adenosine triphosphate (ATP) depletion with glucose/hexokinase and unaffected by treatment with NADase, clotrimazole, or depletion of NAD(P)H with methyl viologen (*23*).

We next studied the HIF-lα recognition sequence in detail. Figure 3A shows alignment of the known or putative pVHL-binding domains

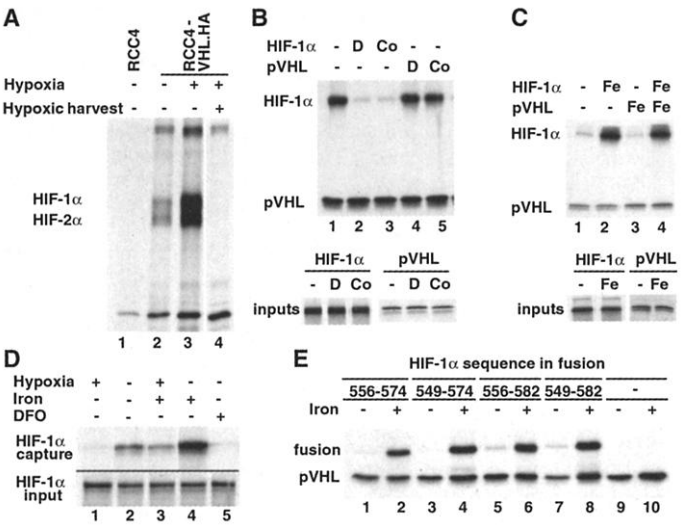

Fig. 1. Regulation of the interaction between pVHL and HIF-α subunits by oxygen and iron availability. (**A**) In vivo coimmunoprecipitation assay. RCC4 cells, which lack pVHL (lane 1), and a stable transfectant expressing HA-tagged pVHL (RCC4/VHL.HA, lanes 2 to 4) were labeled in the presence of the proteasomal inhibitor MG132, either in normoxia (lanes 1 and 2) or hypoxia (lanes 3 and 4) for 4 hours. Cells were lysed either in a hypoxic workstation (lane 4) or on the bench (lanes 1 to 3) (*18*). pVHL and associated proteins were captured with anti-HA. Capture of pVHL and elongins B and C was similar in lanes 2 to 4 (*45*). (**B** to **E**) In vitro assays of the HIFα/pVHL interaction. Labeled HIF-1α and pVHL.HA were generated separately in reticulocyte lysates (IVTT), in the presence or absence of Co(II) (Co), desferrioxamine (D), or added Fe(II) (Fe). Lysates were mixed as indicated, and interactions were assayed by anti-HA immunoprecipitation. (B) Effect of Co(II) or desferrioxamine. (C) Effect of additional Fe(II). (D) Effect of hypoxia. Labeled HIF-1α was generated in IVTT reactions in the presence of desferrioxamine or added Fe(II) either under ambient conditions or in a hypoxic workstation (*19*) and then mixed in the hypoxic workstation with recombinant GST-VBC (*20*). VHL and associated proteins were captured with glutathione-agarose. (E) Fusion proteins containing the indicated HIF-1α sequences, or no insert (lanes 9 and 10), between a Gal4 DNA binding domain and a VP16 transactivation domain, were expressed in reticulocyte lysates with or without added Fe(II). Aliquots were mixed with pVHL.HA and interactions assayed by anti-HA immunoprecipitation.

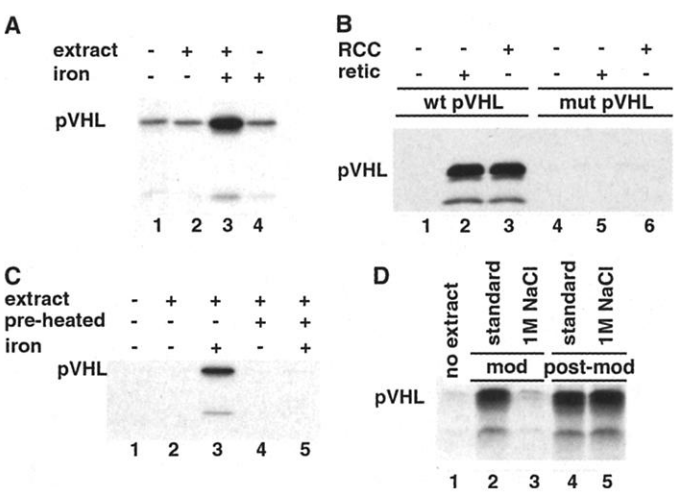

Fig. 2. Analysis of the activity that promotes interaction of HIF with pVHL. Shown are pVHL capture assays in which an unlabeled HIF-1α substrate was immunopurified on beads and washed and aliquots were incubated under different test conditions in buffer or cell extract. After further washing, the beads were assayed for ability to interact with ^{35}S-methionine–labeled pVHL, which was then visualized by fluorography. In (**A**) and (**C**), the HIF substrate was a Gal/HIF/VP16 fusion protein (*21*). In (**B**) and (**D**), the HIF substrate was PK epitope–tagged HIF-1α [HIF-1α(1-826).PK] expressed in insect cells (*22*). (A) Requirement for cell extract. Enhanced capture of pVHL is seen after exposure of the HIF fusion protein to cell extract in the presence of Fe(II). (B) Both RCC4 cell extract and reticulocyte lysate (retic), in the presence of Fe(II), promote the ability of HIF to capture wild-type (wt) but not mutant (mut; Pro86His) pVHL. (C) The Fe(II)-dependent activity of the cell extract is abrogated by preheating at 60°C for 10 min. (D) Addition of NaCl to the RCC4 cell extract (to 1 M final concentration) abrogated the modifying activity (lanes 2 and 3), whereas incubation of the HIF-1α(1-826).PK in NaCl (1 M) after exposure to the cell extract did not alter its ability to capture pVHL (lanes 4 and 5).

among HIF-α homologs, together with the effects of selected point mutations in human HIF-1α on the ability to interact with pVHL. Mutation of a conserved proline, $Pro^{564} \rightarrow Gly$, totally abrogated interaction and $Tyr^{565} \rightarrow Ala$ reduced the interaction, whereas other mutations had little effect or even enhanced interaction. We also used synthetic peptides to inhibit the HIF-lα/pVHL interaction (*24*). A 34-residue peptide encompassing amino acids 549 to 582 did not block interaction. However, the same peptide acquired blocking activity when it was pretreated with mammalian cell extract supplemented with Fe(II) (Fig. 3, B and C). Overall, no peptide could block the interaction without prior enzymatic modification, blocking activity could not be induced by a variety of direct oxidation systems (Fig. 3D), phosphorylation of Tyr^{505} had no effect on the ability of extract to promote blocking activity, and the mutant $Pro^{564} \rightarrow Gly$ peptide did not block the HIF-1α/pVHL interaction, even after exposure to extract.

Prolyl hydroxylation of a HIF-1α degradation domain. Mass spectrometric analyses [matrix-assisted laser desorption ionization–time of flight (TOF)] (*25*) of extract-treated synthetic peptide and recombinant HIF (expressed in insect cells and then treated with mammalian extracts) indicated a series of oxidations as evidenced by +16-dalton mass shifts in ions derived from this sequence. Further analyses by MS/MS (electrospray ionization–quadrupole TOF) revealed oxidations at Pro^{564}, Met^{561}, and Met^{568}. Because these methionines are either nonconserved or can be mutated without effect, methionine oxidation may occur during peptide preparation for MS, and treatments that oxidize methionine efficiently did not mimic the enzymatic activity investigated here (Fig. 3D), we postulated that the critical modification was the oxidation of Pro^{564}.

To test this, we synthesized a peptide (HIF-1α residues 556 to 574) containing a *trans*-4-hydroxy-*S*-proline residue at position 564 (19:Pro564Hyp), because the *trans*-4-hydroxylation is the most common enzymatic proline oxidation (*26*). In striking contrast with previously tested peptides, 19:Pro564Hyp blocked the HIF-1α/pVHL interaction without pretreatment with cell extract (Fig. 4A). Moreover, a biotinylated version of 19:Pro564Hyp specifically bound wild-type but not mutant pVHL, and its ability to bind pVHL was not increased further by incubation with cell extract (*27*) (Fig. 4B).

These results revealed that the enzymatic activity promoting interaction of HIF-1α with pVHL is a prolyl-4-hydroxylase, which we term HIF-α prolyl-hydroxylase (HIF-PH). All previously described prolyl-4-hydroxylases (PHs) are members of the superfamily of 2-oxoglutarate–dependent and related dioxygenases (*28*). Consistent with our data, none of these enzymes have an absolute requirement for ATP or NAD(P), but they do have an absolute requirement for Fe(II) as a cofactor and dioxygen as a cosubstrate (*28*). Structural studies of dioxygenases have defined a nonheme iron center coordinated by an HXD/E. . .H motif (*29*, *30*). The Fe(II) can be readily removed by chelating agents, and enzyme activity is inhibited by substitution of Fe(II) with Co(II) or

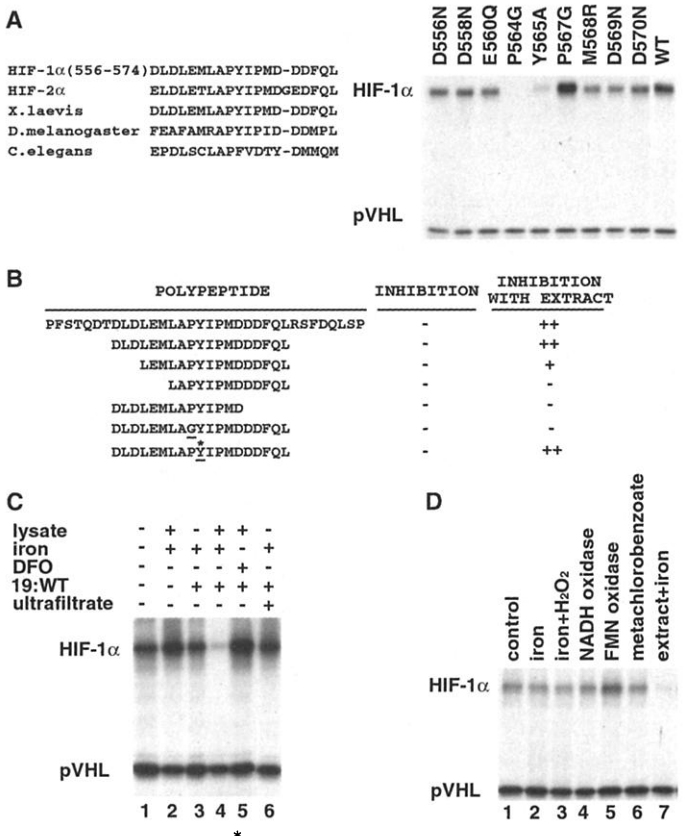

Fig. 3. Analysis of the minimal pVHL-binding domain of HIF-1α. (**A**) (Left) Sequence alignment of the minimal pVHL-binding domain from HIF-1α and HIF-2α, with HIF-α from other organisms (*30*). (Right) Effect of point mutations in the pVHL-binding domain of human HIF-1α. Wild-type (WT) and mutated HIF-1α were generated in reticulocyte lysate and examined for interaction with pVHL.HA by anti-HA immunoprecipitation. (**B**) Summary of the ability of synthetic polypeptides to block the HIF-1α/pVHL interaction before and after exposure to reticulocyte lysate supplemented with Fe(II). Treated and untreated peptides were added to a mixture of HIF-1α and pVHL.HA IVTTs that was then assayed for interaction by anti-HA immunoprecipitation (*24*). Substituted residues are underlined. Ÿ denotes phosphotyrosine. (**C**) Example of a peptide-blocking assay with HIF-1α peptide residues 556 to 574 (19:WT). The peptide blocks the interaction of HIF-1α with pVHL after pretreatment with cell extract in the presence of Fe(II). Peptide pretreated with iron chelated cell extract (DFO), with Fe(II) alone, or with Fe(II) in an ultrafiltrate of cell extract (<5 kD) did not block the interaction. (**D**) Peptide-blocking assay with 19:WT. The assay compares the ability of cell extract and a variety of direct oxidation conditions to block peptide binding. In contrast with exposure to Fe(II)-supplemented cell extract (lane 7), exposure of peptide to Fe(II) (100 μM) alone or with hydrogen peroxide (1 mM), NADH oxidase (1 U/μl) with NADH (1 mM), or NADH-FMN oxidoreductase (7 mU/μl) with NADH (1 mM) or metachlorobenzoic acid (1 mM) did not block peptide binding.

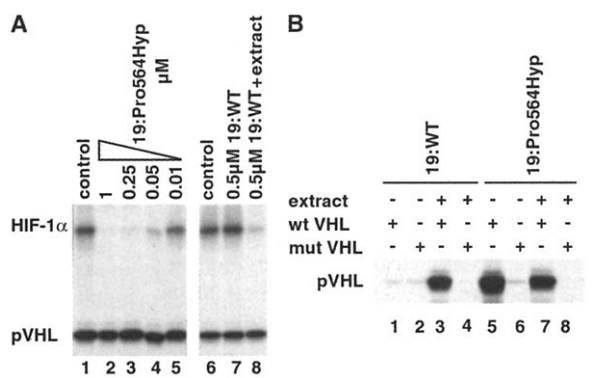

Fig. 4. Hydoxyproline substitution at Pro^{564} promotes interaction of HIF-1α with pVHL. (**A**) Peptide-blocking assay with a HIF peptide (residues 556 to 574) bearing a *trans*-4-hydroxy-*S*-proline substitution at residue Pro^{564}. Blocking peptides were added to a mixture of HIF-1α and pVHL.HA IVTTs that was then assayed for interaction by anti-HA immunoprecipitation. The hydroxyproline substituted peptide (19: Pro564Hyp) inhibits the HIF-1α/pVHL interaction without pretreatment by cell extract (compare lanes 2 to 5 with lane 7). 19:WT, unsubstituted equivalent peptide. Control lanes 1 and 6 demonstrate the interaction with no peptide added. (**B**) pVHL capture assay with biotinylated synthetic peptides. Peptides were assessed for the ability to capture wild-type or mutant ($Pro^{86} \rightarrow His$) pVHL. 19:WT captured pVHL only after incubation with cell extract (lane 3), whereas 19:Pro564Hyp captured wild-type pVHL without pretreatment (lane 5). In both cases, wild-type but not mutant pVHL was captured.

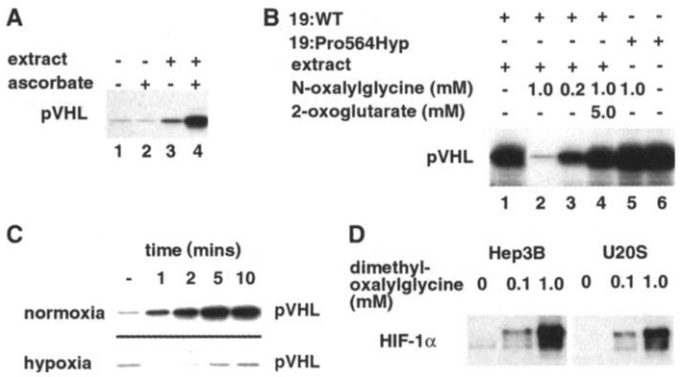

Fig. 5. Effect of cofactors and inhibitors on enzymatic modification of HIF. (**A**) pVHL capture assay with Gal/HIF-1α549-582/VP16 fusion protein as substrate. Ascorbate (2 mM) enhanced the modifying activity of cell extract but had no effect on pVHL capture by the HIF fusion protein in the absence of cell extract. (**B**) pVHL capture assay with biotinylated HIF peptides as substrate. *N*-Oxalylglycine (0.2 to 1 mM) inhibited the modifying activity of cell extract (lanes 2 and 3). Inhibition was overcome by addition of 5 mM 2-oxoglutarate (lane 4). 19:Pro564Hyp captured pVHL efficiently without modification by cell extract (lane 6), and this was not influenced by exposure to *N*-oxalylglycine (lane 5). (**C**) pVHL capture assay with Gal/HIF-1α549-582/VP16 fusion protein as substrate. Effect of hypoxia on the modifying activity of a cell extract. HIF substrate was incubated with cell extract [supplemented with 2 mM ascorbate and 10 μM Fe(II)] for the indicated times at 30°C under ambient conditions or in the hypoxic workstation. The reaction was stopped by washing with DFO, and the HIF substrate was assayed for its ability to interact with pVHL. A time-dependent increase in capture is seen in normoxia, which is greatly reduced in hypoxia. (**D**) HIF-1α immunoblot analysis of extracts of Hep3B and U2OS cells exposed to dimethyl-oxalylglycine for 6 hours. HIF-1α is strongly induced under normoxic culture conditions.

Ni(II) (*26*). Because the known prolyl-4-hydroxylases are dependent on ascorbate for full catalytic activity (*28*), we tested the effect of ascorbate on HIF-PH activity. Ascorbate enhanced HIF-PH, as assessed by the ability of cell extract to modify HIF, but had no effect on HIF in the absence of the extract (Fig. 5A). We next tested a series of 2-oxoglutarate analogs that act as competitive inhibitors of PHs (*31*) for their ability to inhibit HIF-PH. Complete inhibition of modifying activity was observed with *N*-oxalylglycine, and this could be competed by 2-oxoglutarate (Fig. 5B). Similar inhibition, also competed by 2-oxoglutarate, was observed with *N*-oxalyl-2*S*-alanine but not the enantiomer *N*-oxalyl-2*R*-alanine, demonstrating that the effect was not simply due to Fe(II) chelation. We also used a 2-oxoglutarate–dependent dioxygenase, phytanoyl–coenzyme A (CoA) α-hydroxylase (*32*), together with a readily available unnatural substrate (isovaleryl CoA) (*33*) to deplete the cell extract of 2-oxoglutarate produced by the citric acid cycle; as predicted, this prevented the subsequent modification of HIF peptide (*34*).

Because prolyl-4-hydroxylases use molecular oxygen as a cosubstrate, we examined the effect of hypoxia on HIF-PH activity in the presence of maximal supplements of other cofactors. As shown in Fig. 5C, hypoxia markedly suppressed HIF-PH activity, suggesting a direct mechanism for cellular O_2 sensing. Finally, to assess the potential for inhibition of HIF-PH activity to regulate the HIF system in vivo, we synthesized an ester of *N*-oxalylglycine (dimethyl-oxalylglycine) that penetrates cells readily. Exposure of cells to this compound resulted in rapid induction of HIF-1α (Fig. 5D).

In mammalian cells, the best characterized prolyl-4-hydroxylases are the α_1 and α_2 isoforms that modify collagen. These enzymes are reported to have a strict substrate specificity for prolyl residues in collagen repeat sequences, typically $(Pro\text{-}Pro\text{-}Gly)_n$ (*28*). When tested as substrate for recombinant (α1 or α2) human prolyl-4-hydroxylase, the HIF peptide showed no activity (*35*). We therefore postulate that HIF-PH is a previously unknown prolyl-4-hydroxylase.

Discussion. The known properties of 2-oxoglutarate–dependent oxygenases readily explain why the effects of hypoxia on HIF can be mimicked by exposure to iron chelators or cobaltous ions. Two explanations have been advanced previously for these findings. First, it has been proposed that cobaltous ions substitute for ferrous ions at an oxygen sensing iron center (*15*). Because most iron centers (e.g., heme and the large majority of iron sulfur clusters) do not exchange in this way, it was proposed that such a protein must turn over rapidly. Second, it has been postulated that cobaltous ions and iron chelators interfere with Fenton chemistry in nonenzymatic "metal catalyzed oxidation" systems and with signaling through reactive oxygen species (*17, 36*). The labile iron centers associated with prolyl-4-hydroxylases can accommodate the original iron center substitution hypothesis without the need to propose rapid turnover of the sensor. Furthermore, we were unable to promote specific interactions of HIF-α sequences with pVHL by a variety of nonenzymatic oxidation systems. Our results do not exclude the possibility that direct oxidation processes or other oxygen sensing systems impinge on HIF at other sites or indeed on components of the enzymatic prolyl hydroxylation complex. Although our evidence indicates that HIF-PH is distinct from the [α1 and α2] prolyl-4 hydroxylases, it is interesting that these enzymes use protein disulfide isomerase as a β-subunit, thus providing a potential link to sulfhydryl redox chemistry.

The pVHL multiprotein complex belongs to the SCF class of ubiquitin ligases, with pVHL acting as the F-box–like substrate recognition component (*9, 10, 37, 38*). For other F-box proteins, recognition is regulated by substrate phosphorylation. Furthermore, HIF-1α is a phosphoprotein, and phosphorylation has been implicated in HIF regulation (*39, 40*). Although our findings do not exclude HIF-α phosphorylation influencing pVHL binding, they demonstrate a key role for enzymatic hydroxylation of Pro^{564} and thus define a different mechanism of substrate recognition. Furthermore, it is of interest that HIF-α subunits contain other evolutionarily conserved proline residues (*41*). In other studies, we have defined a second subdomain within the NH_2-terminal portion of the HIF-1α oxygen-dependent degradation domain that supports pVHL-dependent ubiquitylation and contains a functionally critical proline residue (*42*), suggesting that similar marking modifications occur elsewhere in HIF-α.

In summary, our results and those of Ivan *et al.* (*43*) suggest that HIF-PH(s) act as a general oxygen sensing mechanism that regulates the HIF transcriptional cascade. It will also be of interest to determine whether hydroxylation occurs at prolines in other molecules, e.g., within so-called "PEST" domains that are associated with rapid protein turnover (*44*). Furthermore, if the prolyl modification is relatively specific to pVHL-mediated ubiquitylation, then it may help define other substrates that are important in pVHL tumor suppressor function.

References and Notes

1. G. L. Semenza, *Genes Dev.* **14**, 1983 (2000).
2. N. V. Iyer *et al.*, *Genes Dev.* **12**, 149 (1997).
3. G. L. Wang, B.-H. Jiang, E. A. Rue, G. L. Semenza, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5510 (1995).
4. L. E. Huang, J. Gu, M. Schau, H. F. Bunn, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7987 (1998).
5. S. Salceda, J. Caro, *J. Biol. Chem.* **272**, 22642 (1997).
6. C. W. Pugh, J. F. O'Rourke, M. Nagao, J. M. Gleadle, P. J. Ratcliffe, *J. Biol. Chem.* **272**, 11205 (1997).
7. M. S. Wiesener *et al.*, *Blood* **92**, 2260 (1998).
8. P. H. Maxwell *et al.*, *Nature* **399**, 271 (1999).
9. K. Iwai *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12436 (1999).
10. J. Lisztwan, G. Imbert, C. Wirbelauer, M. Gstaiger, W. Krek, *Genes Dev.* **13**, 1822 (1999).
11. M. E. Cockman *et al.*, *J. Biol. Chem.* **275**, 25733 (2000).
12. T. Kamura *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10430 (2000).
13. M. Ohh *et al.*, *Nature Cell Biol.* **2**, 423 (2000).
14. K. Tanimoto, Y. Makino, T. Pereira, L. Poellinger, *EMBO J.* **19**, 4298 (2000).
15. M. A. Goldberg, S. P. Dunning, H. F. Bunn, *Science* **242**, 1412 (1988).
16. G. L. Wang, G. L. Semenza, *Blood* **82**, 3610 (1993).
17. G. L. Semenza, *Cell* **98**, 281 (1999).
18. Hypoxia (<0.1% oxygen) was obtained in a worksta-

tion with O_2 and CO_2 and temperature control (Ruskinn Technologies, Leeds, UK). RCC4-VHL.HA, labeling conditions, and coimmunoprecipitation assays have been described previously (*11*). We used 12.5 μM MG132 for proteasomal inhibition. For hypoxic harvest, cells were lysed in the workstation with buffers deoxygenated in the chamber overnight. For standard harvest, the cells were removed from the chamber after hypoxic exposure, before cell lysis.

19. pcDNA3.VHL.HA and pcDNA3.HIF-1α were used to program TNT reticulocyte lysate (Promega). When programming in hypoxia, the reaction mix was preincubated in the workstation for 10 min before addition of the DNA template. An aliquot was removed from the workstation for transcription/translation under ambient oxygenation.
20. Proteins were expressed in rabbit reticulocyte or wheat germ IVTT systems (Promega), in insect cells with the use of a baculoviral system, and in bacteria. IVTTs were programmed with pcDNA3-based vectors encoding subdomains of HIF-1α or pVHL.HA as indicated. For recombinant baculoviral expression, Sf9 insect cells were infected with pFastBac1 vectors (GibcoBRL) encoding PK.HIF-1α(344-698) and HIF-1α(1-826).PK and harvested 60 hours after infection. In bacteria, pVHL was expressed as glutathione-*S*-transferase together with elongins B and C (GST-VBC complex) and HIF-1α as a maltose-binding protein fusion [pMAL.HIF-1α(344-698)].
21. pGal/HIF-1α549-582/VP16 was used to program reticulocyte lysate in the presence of unlabeled methionine. The fusion protein was immunoprecipitated with beads precoated with anti-Gal4 RK5C1 (Santa Cruz). Immunoprecipitated HIF-1α fusion was washed with NETN buffer [50 mM tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 0.5% NP-40], and the beads were incubated with cell lysate in hypotonic extraction buffer [HEB: 20 mM tris (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol] for 60 min at 22°C. The beads were then washed with NETN containing DFO (100 μM) and incubated for 2 hours at 4°C with ^{35}S-methionine–labeled pVHL.HA.
22. HIF-1α(1-826).PK or PK.HIF-1α(344-698) produced from baculoviruses was immunoprecipitated with anti-PK (Serotec). Bead-bound immunoprecipitates were incubated under test conditions and assayed for pVHL.HA capture.
23. D. R. Mole, data not shown.
24. For peptide-blocking assays, peptides were preincubated in cell extract or other conditions for 60 min at 30°C and then added (final concentration, 1 μM) to NETN buffer containing a mixture of HIF-1α and pVHL.HA.
25. Samples for mass spectroscopic analyses were biotinylated synthetic peptides 19:WT (HIF-1α residues 556 to 574) or 34:WT (HIF-1α residues 549 to 582) or baculoviral PK-tagged HIF-1α. After modification by mammalian cell lysates, the material was purified by streptavidin/biotin capture (synthetic peptides) or anti-PK immunoprecipitation and SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (baculoviral HIF). Proteolytic digestion was performed on the beads (synthetic peptides) or in-gel with trypsin and V8 protease at pH 7.8 or V8 protease at pH 4.5. Samples were lyophilized and dissolved in aqueous 0.1% trifluoroacetic acid. Peptides were concentrated, desalted on a 300-μm inside diameter/5-mm length C18 PepMap column (LC Packings, San Francisco, CA), and eluted with 80% acetonitrile. The high-performance liquid chromatography (CapLC, Waters, Milford, MA) was coupled through a Nano-LC inlet to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanoelectrospray Z-spray source. The eluted peptide mixture was analyzed by tandem mass spectrometric sequencing with an automated MS-to-MS/MS switching protocol. Online determination of precursor-ion masses was performed over the *m/z* range from 300 to 1200 atomic mass units in the positive charge detection mode with a cone voltage of 30 V. The collision-induced dissociation for peptide sequencing by MS/MS was performed with argon gas at 20 to 40 eV and a three-dimensional quadrupole resolution.
26. K. I. Kivirikko, R. Myllylä, in *The Enzymology of Post-translational Modification of Proteins*, R. B. Freeman, H. C. Hawkins, Eds. (Academic Press, London, 1980), pp. 53–102.
27. In pVHL capture assays with biotinylated peptides, the peptide was preincubated with cell extract or buffer for 30 min at 30°C and then assayed for ability to bind ^{35}S-labeled pVHL.HA.
28. K. I. Kivirikko, J. Myllyharju, *Matrix Biol.* **16**, 357 (1998).
29. C. J. Schofield, Z. Zhang, *Curr. Opin. Struct. Biol.* **9**, 722 (1999).
30. Single-letter abbreviations for the 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; X, any amino acid; and Y, Tyr.
31. C. J. Cunliffe, T. J. Franklin, N. J. Hales, G. B. Hill, *J. Med. Chem.* **35**, 2652 (1992).
32. G. A. Jansen *et al.*, *J. Lipid Res.* **40**, 2244 (1999).
33. M. Mukherji, M. D. Lloyd, unpublished results.
34. Y.-M. Tian, M. Mukherji, data not shown.
35. Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]glutarate [K. I. Kivirikko, R. Myllylä, *Methods Enzymol.* **82**, 245 (1982)] with recombinant human type I and II prolyl 4-hydroxylases expressed in insect cells. Each reaction contained 0.5 or 1.0 mg of peptide.
36. W. Ehleben, T. Porwol, J. Fandrey, W. Kummer, H. Acker, *Kidney Int.* **51**, 483 (1997).
37. E. E. Patton, A. R. Willems, M. Tyers, *Trends Genet.* **14**, 236 (1998).
38. D. Skowyra, K. L. Craig, M. Tyers, S. J. Elledge, J. W. Harper, *Cell* **91**, 209 (1997).
39. P. W. Conrad, T. L. Freeman, D. Beitner-Johnson, D. E. Millhorn, *J. Biol. Chem.* **274**, 33709 (1999).
40. D. E. Richard, E. Berra, E. Gothie, D. Roux, J. Pouysségur, *J. Biol. Chem.* **274**, 32631 (1999).
41. N. C. Bacon *et al.*, *Biochem. Biophys. Res. Commun.* **249**, 811 (1998).
42. N. Masson, unpublished results.
43. M. Ivan *et al.*, *Science* **292**, 464 (2001).
44. M. Rechsteiner, S. W. Rogers, *Trends Biol. Sci.* **21**, 267 (1996).
45. P. Jaakkola *et al.*, data not shown.
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A Quantum Adiabatic Evolution Algorithm Applied to Random Instances of an NP-Complete Problem

Edward Farhi,[1]* Jeffrey Goldstone,[1] Sam Gutmann,[2] Joshua Lapan,[3] Andrew Lundgren,[3] Daniel Preda[3]

A quantum system will stay near its instantaneous ground state if the Hamiltonian that governs its evolution varies slowly enough. This quantum adiabatic behavior is the basis of a new class of algorithms for quantum computing. We tested one such algorithm by applying it to randomly generated hard instances of an NP-complete problem. For the small examples that we could simulate, the quantum adiabatic algorithm worked well, providing evidence that quantum computers (if large ones can be built) may be able to outperform ordinary computers on hard sets of instances of NP-complete problems.

Although a large quantum computer has yet to be built, the rules for programming such a device, which are derived from the laws of quantum mechanics, are well established. It is already known that quantum computers could solve problems believed to be intractable on classical (i.e., nonquantum) computers. An intractable problem is one that necessarily takes too long to solve when the input gets too big. More precisely, a classically intractable problem is one that cannot be solved using any classical algorithm whose running time grows only polynomially as a function of the length of the input. For example, all known classical factoring algorithms require a time that grows faster than any polynomial as a function of the number of digits in the integer to be factored. Shor's quantum algorithm for the factoring problem (*1*) can factor an integer in a time that grows (roughly) as the square of the number of digits. This raises the question of whether quantum computers could solve other classically difficult prob-

[1]Center for Theoretical Physics, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [2]Department of Mathematics, Northeastern University, Boston, MA 02115, USA. [3]Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*To whom correspondence should be addressed. E-mail: farhi@mit.edu